

#3  
O Birk

11.  Attached:

12.  Preliminary Amendment:

13.  Basic U.S. National fee per Rule 492(a)(1)-(4) was previously timely filed.: February 9, 2001

14. **Calculation of remaining fees due (if any):** based on amended claim(s) per above item

12 (above) or item(s) in PAT-112 (filed previously)     12     14     17     25

15. **CLAIMS FEES**     previously paid     paid herewith as follows:

15A. Small Entity Statement     Herewith     Previously Filed

			Large/Small Entity	Fee Code
16. Total Effective Claims		minus 20 =	x \$18/\$9	+ 966/967
17. Independent Claims		minus 3 =	x \$80/\$40	+ 964/965
18. If any proper multiple dependent claim (ignore improper) is present,			\$270/\$135	+0 968/969
19. Filing Declaration late, fee paid <input type="checkbox"/> previously	<input checked="" type="checkbox"/> now		\$130/\$65	+65 154/254
20.			<b>SUBTOTAL</b>	<b>\$65</b>
21. <b>Original due date:</b>	June 23, 2001			
22. <b>Petition is hereby made</b> to extend the <b>original</b> due date to cover the date this response is filed for which the requisite fee is attached	(1 mo)	\$110/\$55 =	+ 115/215	
	(2mos)	\$390/\$195 =	116/216	
	(3mos)	\$890/\$445 =	117/217	
	(4mos)	\$1390/\$695 =	118/218	
23. If "non-English" box 3 is X'd, add Rule 17(k) processing fee .....		\$130	+ 156	
24. If "assignment" box 6 is X'd, add recording fee. ....		\$40	+0 581	
25.	<b>TOTAL FEE ENCLOSED =</b>			<b>\$65</b>

(Our Deposit Account No. 03-3975)  
 (Our Order No. 82137 | 277848

C# M#

**CHARGE STATEMENT:** The Commissioner is hereby authorized to charge any fee specifically authorized hereafter, or any missing or insufficient fee(s) filed, or asserted to be filed, or which should have been filed herewith or concerning any paper filed hereafter, and which may be required under Rules 16-18 (missing or insufficient fee only) now or hereafter relative to this application and the resulting Official document under Rule 20, or credit any overpayment, to our Account/Order Nos. shown above for which purpose a duplicate copy of this sheet is attached.

This **CHARGE STATEMENT** does not authorize charge of the issue fee until/unless an issue fee transmittal form is filed.

**Pillsbury Winthrop LLP**  
**Intellectual Property Group**

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SE/mll

**NOTE: File in duplicate with PTO receipt (PAT-103A) and attachments**

06/26/2001 MNGUYEN 00000063 09762594

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65.00 OP

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE  
REQUEST FOR FILING NATIONAL PHASE OF  
PCT APPLICATION UNDER 35 U.S.C. 371 AND 37 CFR 1.494 OR 1.495

To: Hon. Commissioner of Patents  
Washington, D.C. 20231



00909

TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)

Atty Dkt: PM 0277848 /PAVA421008  
M# /Client Ref.

From: Pillsbury Winthrop LLP, IP Group: Date: February 9, 2001

This is a **REQUEST** for **FILING** a PCT/USA National Phase Application based on:

1. International Application <u>PCT/US99/18507</u> ↑ country code	2. International Filing Date <u>11 August 1999</u> Day MONTH Year	3. Earliest Priority Date Claimed <u>11 August 1998</u> Day MONTH Year (use item 2 if no earlier priority)
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4. Measured from the earliest priority date in item 3, this PCT/USA National Phase Application Request is being filed within:

(a)  20 months from above item 3 date (b)  30 months from above item 3 date,

(c) Therefore, the due date (unextendable) is February 11, 2001

5. Title of Invention PERIPHERAL-TYPE BENZODIAZEPINE RECEPTOR ASSOCIATED PROTEINS, CLONING, EXPRESSION AND METHODS OF USE

6. Inventor(s) Vassilios Papadopoulos and Hua Li

7. Applicant herewith submits the following under 35 U.S.C. 371 to effect filing:

Please immediately start national examination procedures (35 U.S.C. 371 (f)).

8.  A copy of the International Application as filed (35 U.S.C. 371(c)(2)) is transmitted herewith (file if in English but, if in foreign language, file only if not transmitted to PTO by the International Bureau) including:

a.  Request;  
b.  Abstract;  
c. 57 pgs. Spec. and Claims;  
d. 8 sheet(s) Drawing which are  informal  formal of size  A4  11"

9.  A copy of the International Application has been transmitted by the International Bureau.

10. A translation of the International Application into English (35 U.S.C. 371(c)(2))

a.  is transmitted herewith including: (1)  Request; (2)  Abstract;  
(3) \_\_\_\_\_ pgs. Spec. and Claims;  
(4) \_\_\_\_\_ sheet(s) Drawing which are:  
     informal  formal of size  A4  11"  
b.  is not required, as the application was filed in English.  
c.  is not herewith, but will be filed when required by the forthcoming PTO Missing Requirements  
    Notice per Rule 494(c) if box 4(a) is X'd or Rule 495(c) if box 4(b) is X'd.  
d.  Translation verification attached (not required now).

11.  **PLEASE AMEND** the specification before its first line by inserting as a separate paragraph:  
 a.  --This application is the national phase of international application PCT/US99/18507 filed August 11, 1999 which designated the U.S.--  
 b.  --This application also claims the benefit of U.S. Provisional Application No. 60/096,048, filed August 11, 1998 .--

12.  Amendments to the claims of the International Application **under PCT Article 19 (35 U.S.C. 371(c)(3))**, i.e., before 18th month from first priority date above in item 3, are transmitted herewith (file only if in English) including:

13.  PCT Article 19 claim amendments (if any) have been transmitted by the International Bureau

14.  Translation of the amendments to the claims **under PCT Article 19 (35 U.S.C. 371(c)(3))**, i.e., of **claim amendments** made before 18th month, is attached (required by 20th month from the date in item 3 if box 4(a) above is X'd, or 30th month if box 4(b) is X'd, or else amendments will be considered canceled).

15. **A declaration of the inventor (35 U.S.C. 371(c)(4))**  
 a.  is submitted herewith  Original  Facsimile/Copy  
 b.  is not herewith, but will be filed when required by the forthcoming PTO Missing Requirements Notice per Rule 494(c) if box 4(a) is X'd or Rule 495(c) if box 4(b) is X'd.

16. **An International Search Report (ISR):**  
 a. Was prepared by  European Patent Office  Japanese Patent Office  Other  
 b.  has been transmitted by the international Bureau to PTO.  
 c.  copy herewith (   pg(s).)  plus Annex of family members (   pg(s).).

17. **International Preliminary Examination Report (IPER):**  
 a.  has been transmitted (if this letter is filed after 28 months from date in item 3) in English by the International Bureau with Annexes (if any) in original language.  
 b.  copy herewith in English.  
 c. 1  IPER Annex(es) in original language ("Annexes" are amendments made to claims/spec/drawings during Examination) including attached amended:  
 c. 2  Specification/claim pages #    claims #  
 Dwg Sheets #  
 d.  Translation of Annex(es) to IPER (required by 30<sup>th</sup> month due date, or else annexed amendments will be considered canceled).

18. **Information Disclosure Statement** including:  
 a.  Attached Form PTO-1449 listing documents  
 b.  Attached copies of documents listed on Form PTO-1449  
 c.  A concise explanation of relevance of ISR references is given in the ISR.

19.  **Assignment** document and Cover Sheet for recording are attached. Please mail the recorded assignment document back to the person whose signature, name and address appear at the end of this letter.

20.  Copy of Power to IA agent.

21.  **Drawings** (complete only if 8d or 10a(4) not completed):    sheet(s) per set:  1 set informal;  Formal of size  A4  11"

22. Small Entity Status   is Not claimed  is claimed (pre-filing confirmation required)  
 22(a)    (No.) Small Entity Statement(s) enclosed (since 9/8/00 Small Entity Statements(s) not essential to make claim)

23. **Priority** is hereby claimed under 35 U.S.C. 119/365 based on the priority claim and the certified copy, both filed in the International Application during the international stage based on the filing in (country) the United States of America of:

	Application No.	Filing Date	Application No.	Filing Date
(1)	60/096,048	11 August 1998	(2)	
(3)			(4)	
(5)			(6)	

a.  See Form PCT/IB/304 sent to US/DO with copy of priority documents. If copy has not been received, please proceed promptly to obtain same from the IB.  
 b.  Copy of Form PCT/IB/304 attached.

09/762594

JC05 Rec'd PCT/PTO 09 FEB 2001

8/PRTS

TITLE OF THE INVENTION

Peripheral-type Benzodiazepine Receptor Associated Proteins, Cloning, Expression and Methods of Use

5

Field of the Invention

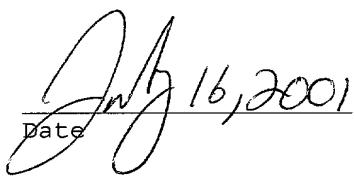
The present invention relates to nucleic acid molecules encoding peripheral-type benzodiazepine receptor (PBR) associated proteins (PAPs), including 10 mutants, variants, fragments and derivatives thereof, and to vectors and host cells comprising such nucleic acid molecules; methods of using PAPs; method for screening for inhibitors and activators of PAPs or PBR; and kits comprising the compositions or 15 polypeptides of the invention.

Background of the Invention

The peripheral-type benzodiazepine receptor (PBR) was originally discovered because it binds the 20 benzodiazepine diazepam with relatively high affinity (Papadopoulos, V. 1993, *Endocr. Rev.* 14:222-240). Benzodiazepines are among the most highly prescribed drugs due to their pharmacological actions in 25 relieving anxiety mediated through modulating the activity of  $\gamma$ -aminobutyric acid receptors in the central nervous system (Costa, E. and Guidotti, A. 1979, *Annu. Rev. Pharmacol. Toxicol.* 19:531-545). PBR is another class of binding sites for benzodiazepines distinct from the aforementioned neurotransmitter 30 receptors. Further studies demonstrated that in addition to benzodiazepines, PBR binds other classes of organic compounds with high affinity (Papadopoulos, 1993, *supra*). PBR, although present in all tissues examined, was found to be particularly high in steroid

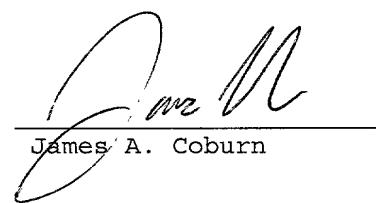
States Code and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Respectfully submitted,

  
JAC 16, 2001  
Date

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8/PRTS  
09/762594

JC05 Rec'd PCT/PTO 09 FEB 2001

TITLE OF THE INVENTIONPeripheral-type Benzodiazepine Receptor Associated Proteins, Cloning, Expression and Methods of Use

5

Field of the Invention

The present invention relates to nucleic acid molecules encoding peripheral-type benzodiazepine receptor (PBR) associated proteins (PAPs), including mutants, variants, fragments and derivatives thereof, and to vectors and host cells comprising such nucleic acid molecules; methods of using PAPs; method for screening for inhibitors and activators of PAPs or PBR; and kits comprising the compositions or 15 polypeptides of the invention.

Background of the Invention

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producing tissues, where it was primarily localized in the outer mitochondrial membrane (OMM) (Anholt, R.R.H. et al. 1986, *J. Biol. Chem.* 261:576-583). An 18 kDa isoquinoline-binding protein was identified as PBR, 5 cloned and expressed (Papadopoulos, V. 1998, *Proc Soc. Exp. Biol. Med.* 217:130-142). It was then demonstrated that PBR is a functional component of the steroidogenic machinery (Papadopoulos, 1998, *supra*; Papadopoulos V. et al. 1990, *J. Biol. Chem.* 265:3772-10 3779) mediating cholesterol delivery from the outer to the inner mitochondrial membrane (Krueger, K. E. and Papadopoulos, V. 1990, *J. Biol. Chem.* 265:15015-15022). Further studies demonstrated that pharmacologically induced reduction of adrenal PBR 15 levels *in vivo* resulted in decreased circulating glucocorticoid levels (Papadopoulos, V. 1998, *supra*). In addition, targeted disruption of the PBR gene in Leydig cells resulted in the arrest of cholesterol transport into mitochondria and steroid formation; 20 transfection of the mutant cells with a PBR cDNA rescued steroidogenesis (Papadopoulos, V. et al. 1997, *J. Biol. Chem.* 272:32129-32135).

PBR is extremely abundant in steroidogenic cells and found primarily on outer mitochondrial membranes 25 (Anholt, R. et al. 1986, *J. Biol. Chem.* 261:576-583). PBR is thought to be associated with a multimeric complex composed of the 18-kDa isoquinoline-binding protein and the 34-kDa pore-forming voltage-dependent anion channel protein, preferentially located on the 30 outer/inner mitochondrial membrane contact sites (McEnery, M. W. et al. *Proc. Natl. Acad. Sci. U.S.A.* 89:3170-3174; Garnier, M. et al. 1994, *Mol. Pharmacol.* 45:201-211; Papadopoulos, V. et al. 1994, *Mol. Cel. Endocrin.* 104:R5-R9). Drug ligands of PBR, upon binding 35 to the receptor, simulate steroid synthesis in

steroidogenic cells *in vitro* (Papadopoulos, V. *et al.* 1990, *J. Biol. Chem.* 265:3772-3779; Ritta, M. N. *et al.* 1989, *Neuroendocrinology* 49: 262-266; Barnea, E. R. *et al.* 1989, *Mol. Cell. Endocr.* 64:155-159; 5 Amsterdam, A. and Suh, B. S. 1991, *Endocrinology* 128:503-510; Yanagibashi, K. *et al.* 1989, *J. Biochem. (Tokyo)* 106: 1026-1029). Likewise, *in vivo* studies showed that high affinity PBR ligands increase steroid plasma levels in hypophysectomized rats (Amri, H. *et al.* 10 1996, *Endocrinology* 137:5707-5718). Further *in vitro* studies on isolated mitochondria provided evidence that PBR ligands, drug ligands, or the endogenous PBR ligand, the polypeptide diazepam-binding inhibitor (BDI) (Papadopoulos, V. *et al.* 1997, 15 *Steroids* 62:21-28), stimulate pregnenolone formation by increasing the rate of cholesterol transfer from the outer to the inner mitochondrial membrane (Krueger, K. E. and Papadopoulos, V. 1990, *J. Biol. Chem.* 265:15015-15022; Yanagibashi, K. *et al.* 1988, 20 *Endocrinology* 123: 2075-2082; Besman, M. J. *et al.* 1989, *Proc. Natl. Acad. Sci. U.S.A.* 86: 4897-4901; Papadopoulos, V. *et al.* 1991, *Endocrinology* 129: 1481-1488).

Based on the amino acid sequence of the 18-kDa 25 PBR, a three dimensional model was developed (Papadopoulos, V. 1996, In: The Leydig Cell. Payne, A. H. *et al.* (eds) Cache River Press, IL, pp 596-628). This model was shown to accomodate a cholesterol molecule and function as a channel, supporting the 30 role of PBR in cholesterol transport. Recently we demonstrated the role of PBR in steroidogenesis by generating PBR negative cells by homologous recombination (Papadopoulos, V. *et al.* 1997, *J. Biol. Chem.* 272:32129-32135) that failed to produce 35 steroids. However, addition of the hydrosoluble

analogue of cholesterol, 22R-hydroxycholesterol, recovered steroid production by these cells, indicating that the cholesterol transport mechanism was impaired. Further cholesterol transport 5 experiments in bacteria expressing the 18-kDa PBR protein provided definitive evidence for a function as a cholesterol channel/transporter (Li and Papadopoulos, 1998, *Endocrinology* 139, 4991-4997).

Studies in a number of tumors such as rat brain 10 containing glioma tumors (Richfield, E. K. et al. 1988, *Neurology* 38:1255-1262), colonic adenocarcinoma and ovarian carcinoma (Katz, Y. et al. 1988, *Eur. J. Pharmacol.* 148: 483-484 and Katz, Y. et al. 1990, *Clinical Sci.* 78:155-158) have shown an abundance of 15 peripheral-type benzodiazepine receptors (PBR) compared to normal tissue. All documents cited herein *infra* and *supra* are hereby incorporated in their entirety by reference thereto. Moreover, a 12-fold increase in PBR density relative to normal parenchyma, 20 was found in human brain glioma or astrocytoma (Cornu, P. et al. 1992, *Acta Neurochir.* 119:146-152). The authors suggested that PBR densities may reflect the proliferative activity of the receptor in these tissues. Recently, the involvement of PBR in cell 25 proliferation was further shown (Neary, J. T. et al. 1995, *Brain Research* 675:27-30; Miettinen, H. et al. 1995, *Cancer Research* 55:2691-2695), and its expression in human astrocytic tumors was found to be associated with tumor malignancy and proliferative 30 index (Miettinen, H. et al. *supra*; Alho, H. 1994, *Cell Growth Different.* 5:1005-1014). Characterization of PBR in human breast cancer biopsies, led to the discovery that the invasive and metastatic ability of human breast tumor cells is proportional to the level 35 of PBR expressed, and correlates with the subcellular

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localization of PBR in these cells in that PBR is found primarily in the nucleus in aggressive tumor cells whereas PBR is found primarily in the cytoplasm of invasive but non-aggressive cells. These changes 5 in PBR expression can be used as a tool for detection, diagnosis, prevention and treatment in breast cancer patients, in particular, and in aggressive solid tumors in general.

Since both PBR and its endogenous ligand, the 10 polypeptide diazepam binding inhibitor, are constitutively expressed in steroidogenic cells, the regulation of PBR function by hormones may be due to its association with other proteins. This interaction may result in the initiation of steroid biosynthesis. 15 Therefore, there is a need to identify proteins which associate with PBR and may modulate PBR function.

#### **Summary of the Invention**

The present invention meets the need described above. We have identified PBR associated proteins (PAPs) that 20 interact with PBR using the two-hybrid system. We used PBR as a bait to screen a mouse testis cDNA library. Five clones were isolated by their ability to interact with PBR: PAP3, PAP7, PAP8, PAP15, and PAP20. Among the nucleotide sequences identified, 25 PAP3 was identical to the previously isolated meg1 protein (Don, J. and Wolgemuth, D. J., 1992, *Cell Growth Differ.* 3, 495; Ever, L. et al., 1999, *Cell Growth Differ.* 10, 19-26). PAP7, PAP8, PAP15, and PAP20 are novel sequences according to a search in the 30 Genebank database which did not find a match for these sequences. PAP7 and PAP17 are different clones of the same novel protein product. All PAPs have fatty acylation (myristoylation) sites and PKC

phosphorylation sites. In addition, PAP20 has a PKA phosphorylation site. The distribution and function of the PAPs, as well as their functional relationship to PBR is under investigation.

5 So far, the distribution of PAP7 in major mouse tissues such as brain, testis, ovary, adrenal, kidney and muscle showed a profile similar to the broader expression pattern of PBR, with an expression level paralleling the steroidogenic ability of the tissue.

10 These data imply a role for these PAPs in the regulation of PBR function, serving as endogenous ligands or allosteric modulators of the receptor.

Therefore, it is an object of the present invention to provide novel DNA fragments encoding PBR 15 associated proteins, PAP3 (SEQ ID NO:1), PAP7 (SEQ ID NO:2) and Genbank accession no. AF022770, PAP8 (SEQ ID NO:3), PAP15 (SEQ ID NO:4), and PAP20 (SEQ ID NO:5) GenBank accession no. AF020338. The DNA fragment is useful as a diagnostic agent for detection of nucleic 20 acid sequences encoding PBR-associated proteins, as an agent for preparation of the protein encoded by the DNA, for the preparation of sequences encoding PAPs, whether cDNA or genomic, and as therapeutic agents.

It is another object of the invention to provide 25 an amino acid sequence for PAPs encoded by the DNA sequences described above.

It is another object of the invention to provide a recombinant vector comprising a vector and the above described DNA fragments.

30 It is a further object of the present invention to provide a host cell transformed with the above-described recombinant DNA construct.

It is another object of the present invention to provide a method for producing PAPs which comprises culturing a host cell under conditions such that the above-described DNA fragment is expressed and a PAP is thereby produced, and isolating the PAP for use as a reagent, for example for screening drugs and inhibitors of PBR or the PAP itself, for diagnosis, and for therapy.

It is a further object of the present invention to provide an antibody to the above-described recombinant PAPs.

It is yet another object of the present invention to provide a method for detecting any of PAP3, PAP7, PAP8, PAP15, or PAP20 in a sample comprising:

(i) contacting a sample with antibodies which recognize any one of the PAPs mentioned above; and  
(ii) detecting the presence or absence of a complex formed between the PAP and antibodies specific therefor.

It is a further object of the present invention to provide a diagnostic kit comprising an antibody against PAP and ancillary reagents suitable for use in detecting the presence of PAP in cells, tissue or serum from yeast, mammals, animals, birds, fish, and plants.

It is yet another object of the present invention to provide a method for the detection of PAP from a sample using the polymerase chain reaction.

It is a further object of the present invention to provide a diagnostic kit comprising primers or oligonucleotides specific for PAP RNA or cDNA suitable for hybridization to PAP RNA or cDNA and/or amplification of PAP sequences and ancillary reagents suitable for use in detecting PAP RNA/cDNA in mammalian tissue.

It is yet another object of the present invention to provide a method for the detection of a PAP in a sample which comprises assaying for the presence or absence of PAP RNA or cDNA in a sample by hybridization assays.

It is an object of the present invention to provide a method for the measurement of PBR in a sample. The method comprises measuring the presence of a PAP complexed with PBR.

It is yet another object of the present invention to provide a method for modulating the function or altering the targeting of PBR by increasing or decreasing interaction of PBR with a PAP. PBR functions which can be modulated include cholesterol transport into the cell, steroid production, cell proliferation, and embryogenesis.

It is still another object of the present invention to provide a method for increasing or decreasing PBR function or expression in a cell by providing into the cell a PAP such that PBR function is increased or decreased.

It is yet another object of the present invention to provide a method for increasing or reducing steroidogenesis by altering the level of PAP in said cell.

It is still another object of the invention to provide a therapeutic method for the treatment or amelioration of diseases resulting from increased cell proliferation resulting from aberrant PBR function or expression or localization, said method comprising providing to an individual in need of such treatment an effective amount of a PAP, in a pharmaceutically acceptable diluent, of a PAP such that the aberrant PBR expression, function or localization, is corrected.

It is another object of the present invention to provide a therapeutic method for the treatment or amelioration of diseases resulting from decreased cell proliferation, said method comprising providing to an 5 individual in need of such treatment an effective amount of PAP or an antibody against PAP or an agent which inhibits PAP expression or function in a pharmaceutically acceptable excipient.

It is another object of the present invention to 10 provide a therapeutic method for the treatment or amelioration of diseases resulting from decreased or increased steroidogenesis, said method comprising providing to an individual in need of such treatment an effective amount of a PAP or an antibody against 15 PAP or an agent which inhibits or activates PAP expression or function in a pharmaceutically acceptable diluent.

It is yet a further object of the present invention to provide a cDNA sequence encoding PAPs and 20 vectors incorporating all or a fragment of said sequence, and cells, prokaryotic and eukaryotic, transformed or transfected with said vectors, for use in screening agents and drugs which inhibit expression or function of PAPs or PBR in such cells.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and 30 appended claims, and accompanying drawings where:

**Figure 1.** PAP7 protein expressed in MA10 mouse Leydig tumor cells. (A) Western blot. (B), Immunocytochemistry.

**Figure 2.** PAP7 mRNA Tissue distribution 35 analysis by Dot blot analysis. (A), a Master blot

PCT/US99/18507

containing 100-500 ng of poly(A)+ RNA from mouse tissues were hybridized at high stringency with a <sup>32</sup>P-labeled PAP7 probe as described under "Materials and Methods." The autoradiogram was exposed overnight.

5 (B), densitometric analysis of PAP7 expression.

**Figure 3.** PAP7 tissue distribution analysis by Northern blot analysis. (A), Northern blot analysis was performed using 20  $\mu$ g of total RNA/lane from different mouse tissues as indicated. The blot was 10 hybridized at high stringency with a <sup>32</sup>P-labeled PAP7 probe as described under "Materials and Methods". The autoradiogram was exposed overnight. (B), Densitometric analysis of PAP7 expression.

**Figure 4.** PBR tissue distribution analysis by 15 Northern blot analysis. (A), Northern blot analysis was performed using 20  $\mu$ g of total RNA/lane from different mouse tissues as indicated. The blot was hybridized at high stringency with a <sup>32</sup>P-labeled PBR probe as described under "Materials and Methods". The 20 autoradiogram was exposed overnight. (B), For Northern blot analysis, blots were quantitated by densitometry.

**Figure 5.** Immunohistological staining of mouse tissues with anti-PAP7 antibody.

25 **Figure 6.** The effect of PAP7 on Steroid Biosynthesis. Progesterone formation in MA-10 stimulated by hCG (A), for different time course (B). The results shown represent the means + S.D. from 2 to 6 independent experiments.

30 **Figure 7.** PAP3 mRNA Tissue distribution analysis by Dot blot analysis. (A), a Master blot containing 100-500 ng of poly(A)+ RNA from mouse tissues were hybridized at high stringency with a <sup>32</sup>P-labeled PAP3 probe as described under "Experimental

Procedures." The autoradiogram was exposed overnight. (B), densitometric analysis of PAP3 expression.

**Figure 8.** PAP20 mRNA Tissue distribution analysis by Dot blot analysis. (A), a Master blot 5 containing 100-500 ng of poly(A)+ RNA from mouse tissues were hybridized at high stringency with a <sup>32</sup>P-labeled PAP20 probe as described under "Experimental Procedures." The autoradiogram was exposed overnight. (B), densitometric analysis of PAP20 expression.

10 **DETAILED DESCRIPTION**

The five PAPs described in this application were discovered using a two-hybrid assay. The two-hybrid assay is a yeast-based genetic assay used to detect protein-protein interactions *in vivo*. A positive 15 result obtained with the two-hybrid assay allows rapid identification of genes encoding proteins that interact with a target protein. In addition the two-hybrid assay is a sensitive method for detecting weak and transient interactions, which are probably the 20 norm in large native complexes. Most notably, because the two-hybrid assay is performed *in vivo*, the proteins involved are more likely to be in their native conformations.

The two-hybrid assay is based on the fact that 25 many eukaryotic transcriptional activators consist of two physically separable modular domains: one acts as the DNA-binding domain, while the other functions as the transcriptional activation domain. The DNA-binding domain localizes the transcription factor to 30 specific DNA sequences present in the upstream region of genes that are regulated by this factor, while the activation domain contacts other components of the transcription machinery required to initiate transcription. Both domains are required for normal

activation functioning, and normally the two domains are part of the same protein.

In our PAPs screening experiment, the MATCHMAKER Two-Hybrid System from CLONTECH was used. In the 5 MATCHMAKER System, sequences encoding the two functional domains of the GAL4 transcriptional activator have been cloned into two different shuttle/expression vectors (pGBT9 and pGAD10). The pGBT9 hybrid cloning vector is used to generate a 10 fusion of the GAL4 DNA-binding domain with PBR protein. The pGAD10 hybrid cloning vector is used to generate a fusion of the GAL4 activation domain with a collection of random proteins in a fusion mouse testis library (CLONTECH). Both hybrid proteins are targeted 15 to the yeast nucleus by nuclear localization sequences that either are an intrinsic part of the GAL4 DNA-binding domain or have been added to the activation domain from a heterologous source. If PBR protein and an unknown protein or proteins interact with each 20 other, the DNA-binding domain of GAL4 will be tethered to its transcriptional activation domain, and the proper function of the transcription of an appropriate reporter gene (lacZ or HIS3) containing upstream GAL4 binding sites is used to indicate interaction between 25 the two proteins. This allows a positive selection for clones that are transformed by two interacting hybrid constructs and makes library screening more convenient and practical. After a positive clone has been identified, the gene corresponding to the interacting 30 protein was sequenced using the sequencing primers provided in the kit.

In one embodiment, the present invention relates to a DNA or cDNA sequence encoding PBR associated proteins (PAPs). Five clones were isolated, PAP3, 35 comprising 568 bp and identified in SEQ ID NO:1 (Don,

J. and Wolgemuth, D. J., 1992, *Cell Growth Differ.* 3, 495; Ever, L. et al., 1999, *Cell Growth Differ.* 10, 19-26 ) which encodes a peptide of 83 amino acids identified in SEQ ID NO:6; PAP7, comprising 577 bp extending from 696 to 1164 of the sequence identified in SEQ ID NO:2, which encodes a polypeptide of 363 amino acids, identified in SEQ ID NO:7; PAP8, comprising 568 bp identified in SEQ ID NO:3, which encode a polypeptide of 190 amino acids, identified in SEQ ID NO:8; PAP15 comprising 490 bp identified in SEQ ID NO:4, which encode a polypeptide of 164 amino acids, identified in SEQ ID NO:9; and PAP20 comprising 588 bp identified in SEQ ID NO:5, which encode a polypeptide of 196 amino acids, identified in SEQ ID NO:10.

PAP3 has been identified as the previously isolated meg 1 protein.

PAP7 and PAP17 are different clones of the same novel protein product. Additional PAP7 sequence has been obtained using the 5', 3'RACE system (CLONTECH) and the near full-length gene is identified in SEQ ID NO:2 including the stop codon and some untranslated sequence at the 3' end. The polypeptide encoded by the DNA sequence would have a calculated molecular weight of about 50 kD. Using an PAP7 antibody produced from the initial isolated DNA fragment of 577 bp, a protein of about 52 kD is immunoprecipitated as shown in the Examples below. Analysis of the protein sequence indicates several consensus sequences and important sites such as: two potential myristylation sites at positions 262-267 and 271-276 of SEQ ID NO:7 and five PKC phosphorylation sites at 395-396, 113-115, 255-257, 280-282, 331-333, and 339-341 of SEQ ID NO:7, an Acyl-Co-A site at position 24-108 of SEQ ID NO:7, a nuclear localization domain at position 150-

167 of SEQ ID NO:7, a troponin site at position 98-247  
of SEQ ID NO:2 and an HSP90 domain at position 126-155  
of SEQ ID NO:7. The distribution and expression of  
PAP7 were examined in major mouse tissues such as  
5 brain, testis, ovary, adrenal, and kidney, as well as  
in tissue culture cell lines such as mouse C6 glioma  
cells, MA-10 Leydig cells, and Y1 adrenal cortical  
cells. The PAP7 expression pattern is similar to the  
broader expression profile of PBR in both tissues and  
10 cell lines involved in steroid biosynthesis.

Additionally, both PBR and PAP7 expression level in  
the cell lines correlated with their steroidogenic  
biosynthesis ability, which suggests that PAP7 may be  
involved in steroid biosynthesis through PBR.

15 PAP 8, PAP15, and PAP20, are novel genes. The  
polypeptide encoded by PAP20 has two potential  
myristoylation sites, one PKC phosphorylation site and  
one PKA phosphorylation site. Protein myristoylation  
enables the protein to attach to the cellular membrane  
20 and thus take part in cell signaling (Casey, P.J.  
1995, *Science* 268, 221-225; Boutin, J. A. 1997, *Cell  
Signal* 9, 15-35). PAP20 is predominantly expressed in  
the testis. Interaction of PBR with PAP20 increased  
the affinity of ligand binding using PK11195 as a  
25 ligand. Therefore, it is likely that PAP20 serves to  
increase or decrease PBR function by modulating PBR's  
affinity to its endogenous ligand, DBI. The tissue  
distribution of PAP3 and PAP20 is shown in Figure 7  
and Figure 8, respectively.

30 Thus, one aspect of the invention provides an  
isolated nucleic acid molecule comprising a  
polynucleotide having a nucleotide sequence selected  
from SEQ ID NO:1-9 encoding PAPs polypeptides. It is  
within the skill of a person with ordinary skill in  
35 the art to use the sequences provided herein for the

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purpose of cloning cDNA or genomic sequences which encode other parts or complete portions of the PAP genes described herein and therefore, these related sequences are encompassed within the present

5 invention.

In addition, isolated nucleic acid molecules of the invention include DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode PAPs. Of course, the genetic code and species-specific codon preferences are well known in the art. Thus, it would be routine for one skilled in the art to generate the degenerate variants described above, for instance, to optimize codon 10 expression for a particular host (e.g., change codons in the human mRNA to those preferred by a bacterial host such as *E.coli* or plant host).

15

Nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the 20 form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non- 25 coding strand, also referred to as the antisense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For 30 example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or 35 substantially) DNA molecules in solution. Isolated

RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such 5 molecules produced synthetically.

The present invention is further directed to nucleic acid molecules encoding portions or fragments of the nucleotide sequences described herein.

Fragments include portions of the nucleotide sequences 10 of at least 10 contiguous nucleotides in length selected from any two integers, one of which representing a 5' nucleotide position and a second of which representing a 3' nucleotide position, where the first nucleotide for each nucleotide sequence is 15 position 1. That is, every combination of a 5' and 3' nucleotide position that a fragment at least 10 contiguous nucleotide bases in length or any integer between 10 and the length of an entire nucleotide sequence minus 1.

20 Further, the invention includes polynucleotides comprising fragments specified by size, in nucleotides, rather than by nucleotide positions. The invention includes any fragment size, in contiguous nucleotides, selected from integers between 1- and the 25 entire length of an entire nucleotide sequence minus 1. Preferred sizes include 20-50 nucleotides, 50-300 nucleotides useful as primers and probes. Regions from which typical sequences may be derived include but are not limited to, for example, regions encoding 30 specific epitopes or domains within said sequence, for example, the PBR binding domain extending in SEQ ID NO:1, 2, 3, 4, and 5, potential myristylation sites at positions 262-267 and 271-276 of SEQ ID NO:7 and five PKC phosphorylation sites at 395-396, 113-115, 35 255-257, 280-282, 331-333, and 339-341 of SEQ ID NO:7,

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an Acyl-Co-A site at position 24-108 of SEQ ID NO:7, a nuclear localization domain at position 150-167 of SEQ ID NO:7, a troponin site at position 98-247 of SEQ ID NO:7 and an HSP90 domain at position 126-155 of SEQ ID NO:7, among others.

In another aspect, the invention provides isolated nucleic acid molecules comprising polynucleotides which hybridize under stringent hybridization conditions to a polynucleotide sequence of the present invention described above, or a specified fragment thereof. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5X SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 g/ml denatured sheared salmon sperm DNA, followed by washing the filters in 0.1X SSC at about 65°C.

The sequences encoding the polypeptides of the present invention or portions thereof may be fused to other sequences which provide additional functions known in the art such as a marker sequence, or a sequence encoding a peptide which facilitates purification of the fused polypeptide, peptides having antigenic determinants known to provide helper T-cell stimulation, peptides encoding sites for post-translational modifications, or amino acid sequences which target the fusion protein to a desired location, e.g. a heterologous leader sequence.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the PAPs of the present invention. A variant may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of

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several alternate forms of a gene occupying a given locus of a chromosome of an organism. Non-naturally occurring variants may be produced by known mutagenesis techniques. Such variants include those produced by 5 nucleotide substitution, deletion, or addition of one or more nucleotides in the coding or noncoding regions or both. Alterations in the coding regions may produce conservative or nonconservative amino acid substitutions, deletions, or additions. Especially 10 preferred among these are silent substitutions, additions, and deletions which do not alter the properties and activities of PAPs polypeptides disclosed herein or portions thereof. Also preferred in this regard are conservative substitutions.

15 Nucleic acid molecules with at least 90-99% identity to a nucleic acid identified above is another aspect of the present invention. These nucleic acids are included irrespective of whether they encode a polypeptide having PAP activity. By "a polypeptide 20 having PAP activity" is intended polypeptides exhibiting activity similar, but not identical, to an activity of the PAP of the invention, as measured in the assays described below. The biological activity or function of the polypeptides of the present 25 invention are expected to be similar or identical to polypeptides from other organisms that share a high degree of structural identity/similarity.

In another embodiment, the present invention relates to a recombinant DNA molecule that includes a 30 vector and a DNA sequence as described above. The vector can take the form of a plasmid, phage, cosmid, YAC, eukaryotic expression vector such as a DNA vector, *Pichia pastoris*, or a virus vector such as for example, baculovirus vectors, retroviral vectors or 35 adenoviral vectors, and others known in the art. The

cloned gene may optionally be placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences, or sequences which may be inducible and/or cell type-specific.

5 Suitable promoters will be known to a person with ordinary skill in the art. The expression construct will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation.

10 Among the vectors preferred for use include pGBT9, PGAD10 (Clonetech), PSVzeo (Invitrogen), pBlueScript (Stratagene), pCMV5 (Invitrogen), pCRII (Invitrogen) to name a few.

15 Introduction of the construct into the host cell can be effected by calcium phosphate transfection, electroporation, infection, and other methods known in the art and described in standard laboratory manuals such as Current Protocols in Molecular Biology, Ausubel, F. M. et al. (Eds), Wiley & Sons, Inc. All 20 documents cited herein supra and infra are hereby incorporated in their entirety by reference thereto.

25 In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-described recombinant DNA constructs. The host cell can be prokaryotic (for example, bacterial), lower eukaryotic (for example, yeast or insect) or higher eukaryotic (for example, all mammals, including but not limited to rat and human). Both prokaryotic and eukaryotic host cells 30 may be used for expression of desired coding sequences when appropriate control sequences which are compatible with the designated host are used. Among prokaryotic hosts, *E. coli* is most frequently used. Expression control sequences for prokaryotes include 35 promoters, optionally containing operator portions,

and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts are commonly derived from, for example, pBR322, a plasmid containing operons conferring ampicillin and tetracycline 5 resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful transformants by selection. Please see e.g., Maniatis, Fitsch and Sambrook, Molecular 10 Cloning; A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a sequence encoding an IgG molecule, an adjuvant, a carrier, or an agent for 15 aid in purification of PAPs, such as glutathione S-transferase, or a series of histidine residues also known as a histidine tag. The recombinant molecule can be suitable for transfecting plant cells or eukaryotic cells, for example, mammalian cells and 20 yeast cells in culture systems. *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, and *Pichia pastoris* are the most commonly used yeast hosts, and are convenient fungal hosts. Control sequences for yeast vectors are known in the art. Mammalian cell 25 lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), such as HEK293 cells, and NIH 3T3 cells, MA10 Leydig cells, mouse C6 glioma cells, Y1 adrenal cells, 30 and breast cancer cell lines such as MDA-231, MCF-7, to name a few. Suitable promoters are also known in the art and include viral promoters such as that from SV40, Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV), and cytomegalovirus

(CMV). Mammalian cells may also require terminator sequences and poly A addition sequences; enhancer sequences which increase expression may also be included, and sequences which cause amplification of the gene may also be desirable. These sequences are known in the art. The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the protein described below.

In another embodiment, the present invention relates to the PAP polypeptides described above or any allelic variation thereof which is immunologically identifiable with the polypeptides.

A polypeptide or amino acid sequence derived from the amino acid sequences mentioned above, refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 2-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence.

A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid sequence; it may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system. In addition the polypeptide can be fused to other proteins or polypeptides which increase its antigenicity, such as adjuvants for example.

As noted above, the methods of the present invention are suitable for production of any

polypeptide of any length, via insertion of the above-described nucleic acid molecules or vectors into a host cell and expression of the nucleotide sequence encoding the polypeptide of interest by the host cell.

5 Introduction of the nucleic acid molecules or vectors into a host cell to produce a transformed host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction,

10 infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis et al., Basic Methods In Molecular Biology (1986). Once transformed host cells have been obtained, the cells may be cultivated under any

15 physiologically compatible conditions of pH and temperature, in any suitable nutrient medium containing assimilable sources of carbon, nitrogen and essential minerals that support host cell growth.

Recombinant polypeptide-producing cultivation

20 conditions will vary according to the type of vector used to transform the host cells. For example, certain expression vectors comprise regulatory regions which require cell growth at certain temperatures, or addition of certain chemicals or inducing agents to

25 the cell growth medium, to initiate the gene expression resulting in the production of the recombinant polypeptide. Thus, the term "recombinant polypeptide-producing conditions," as used herein, is not meant to be limited to any one set of cultivation

30 conditions. Appropriate culture media and conditions for the above-described host cells and vectors are well-known in the art. Following its production in the host cells, the polypeptide of interest may be isolated by several techniques. To liberate the

35 polypeptide of interest from the host cells, the cells

are lysed or ruptured. This lysis may be accomplished by contacting the cells with a hypotonic solution, by treatment with a cell wall-disrupting enzyme such as lysozyme, by sonication, by treatment with high pressure, or by a combination of the above methods. Other methods of bacterial cell disruption and lysis that are known to one of ordinary skill may also be used.

Following disruption, the polypeptide may be separated from the cellular debris by any technique suitable for separation of particles in complex mixtures. The polypeptide may then be purified by well known isolation techniques. Suitable techniques for purification include, but are not limited to, ammonium sulfate or ethanol precipitation, acid extraction, electrophoresis, immunoadsorption, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, immunoaffinity chromatography, size exclusion chromatography, liquid chromatography (LC), high performance LC (HPLC), fast performance LC (FPLC), hydroxylapatite chromatography and lectin chromatography.

25 The recombinant polypeptide or fusion protein can be used, detectably labeled and unlabeled, as a diagnostic tool for the detection of PAPs or for the detection and measurement of PBR. Additionally, these polypeptides can be used in a method for modulating  
30 PBR expression. In addition, the recombinant protein can be used as a therapeutic agent to reduce cell death and/or increase cell proliferation via its effect on PBR function. The transformed host cells can be used to analyze the effectiveness of drugs and  
35 agents which modulate PBR function, expression or

targeting via their effect on the expression or function of PAPs, such as host proteins or chemically derived agents or other proteins which may interact with the cell to alter the PAP function or expression, 5 thereby modulating PBR function, expression or localization.

In another embodiment, the present invention relates to monoclonal or polyclonal antibodies specific for the above-described recombinant proteins 10 (or polypeptides). For instance, an antibody can be raised against a peptide described above, or against a portion thereof of at least 10 amino acids, preferably, 11-15 amino acids. Persons with ordinary skill in the art using standard methodology can raise 15 monoclonal and polyclonal antibodies to the protein (or polypeptide) of the present invention, or a unique portion thereof. Material and methods for producing antibodies are well known in the art (see for example Goding, in, Monoclonal Antibodies: Principles and 20 Practice, Chapter 4, 1986).

The amount of PAP expression can be detected at several levels. Using standard methodology well known in the art, assays for the detection and quantitation of PAP RNA can be designed, and include northern 25 hybridization assays, *in situ* hybridization assays, and PCR assays, among others. Please see e.g., Maniatis, Fitch and Sambrook, Molecular Cloning: A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985), or Current Protocols in 30 Molecular Biology, Ausubel, F. M. et al. (Eds), Wiley & Sons, Inc. for general description of methods for nucleic acid hybridization. Polynucleotide probes for the detection of PAP RNA can be designed from the sequences described in SEQ ID NO: 1-9. For example,

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RNA isolated from samples can be coated onto a surface such as a nitrocellulose membrane and prepared for northern hybridization. In the case of *in situ* hybridization of biopsy samples for example, the 5 tissue sample can be prepared for hybridization by standard methods known in the art and hybridized with polynucleotide sequences which specifically recognize PAP RNA. The presence of a hybrid formed between the sample RNA and the polynucleotide can be detected by 10 any method known in the art such as radiochemistry, or immunochemistry, to name a few.

One of skill in the art may find it desirable to prepare probes that are fairly long and/or encompass regions of the amino acid sequence which would have a 15 high degree of redundancy in the corresponding nucleic acid sequences. In other cases, it may be desirable to use two sets of probes simultaneously, each to a different region of the gene. While the exact length of any probe employed is not critical, typical probe 20 sequences are no greater than 500 nucleotides, even more typically they are no greater than 250 nucleotides; they may be no greater than 100 nucleotides, and also may be no greater than 75 nucleotides in length. Longer probe sequences may be 25 necessary to encompass unique polynucleotide regions with differences sufficient to allow related target sequences to be distinguished. For this reason, probes are preferably from about 10 to about 100 nucleotides in length and more preferably from about 30 20 to about 50 nucleotides.

The DNA sequence of PAPs can be used to design primers for use in the detection of PAPs using the polymerase chain reaction (PCR) or reverse transcription PCR (RT-PCR). The primers can 35 specifically bind to the PAP cDNA produced by reverse

transcription of PAP RNA, for the purpose of detecting the presence, absence, or quantifying the amount of PAP by comparison to a standard. The primers can be any length ranging from 7-40 nucleotides, preferably 10-15 nucleotides, most preferably 18-25 nucleotides homologous or complementary to a region of the PAP sequence. Reagents and controls necessary for PCR or RT-PCR reactions are well known in the art. The amplified products can then be analyzed for the presence or absence of PAP sequences, for example by gel fractionation, by radiochemistry, and immunochemical techniques. This method is advantageous since it requires a small number of cells. Once PAP is detected, a determination whether the cell is overexpressing or underexpressing PAP can be made by comparison to the results obtained from a normal cell using the same method. For example, increased PAP7 RNA levels correlate with PBR expression levels, especially in steroidogenic cells, wherein, an increase in steroidogenic capability of the cells correlates with an increase in PBR and PAP7 RNA.

In another embodiment, the present invention relates to a diagnostic kit for the detection of PAP RNA in cells, said kit comprising a package unit having one or more containers of PAP oligonucleotide primers for detection of PAP by PCR or RT-PCR or PAP polynucleotides for the detection of PAP RNA in cells by *in situ* hybridization or northern analysis, and in some kits including containers of various reagents used for the method desired. The kit may also contain one or more of the following items: polymerization enzymes, buffers, instructions, controls, detection labels. Kits may include containers of reagents mixed together in suitable proportions for performing the

methods in accordance with the invention. Reagent containers preferably contain reagents in unit quantities that obviate measuring steps when performing the subject methods.

5 In a further embodiment, the present invention provides a method for identifying and quantifying the level of PAP present in a particular biological sample. Any of a variety of methods which are capable of identifying (or quantifying) the level of PAP in a  
10 sample can be used for this purpose.

Diagnostic assays to detect PAPs may comprise a biopsy or *in situ* assay of cells from an organ or tissue sections, as well as an aspirate of cells from a tumor or normal tissue. In addition, assays may be  
15 conducted upon cellular extracts from organs, tissues, cells, urine, or serum or blood or any other body fluid or extract.

When assaying a biopsy, the assay will comprise, contacting the sample to be assayed with a PAP ligand, natural or synthetic, or an antibody, polyclonal or monoclonal, which recognizes PAP, or antiserum capable of detecting PAP, and detecting the complex formed between PAP present in the sample and the PAP ligand or antibody added.

25 PAP ligands or substrates include for example, PBR, in addition to natural and synthetic classes of ligands and their derivatives which can be derived from natural sources such as animal or plant extracts.

30 PAP ligands or anti-PAP antibodies, or fragments of ligand and antibodies capable of detecting PAP may be labeled using any of a variety of labels and methods of labeling for use in diagnosis and prognosis of disease associated with increased cell proliferation, such as cancer, or reduced cell death.  
35 Examples of types of labels which can be used in the

present invention include, but are not limited to, enzyme labels, radioisotopic labels, non-radioactive isotopic labels, and chemiluminescent labels.

Examples of suitable enzyme labels include malate 5 dehydrogenase, staphylococcal nuclease, delta-5- steroid isomerase, yeast-alcohol dehydrogenase, alpha-glycerol phosphate dehydrogenase, triose phosphate 10 isomerase, peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, 15 ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, acetylcholine esterase, etc.

Examples of suitable radioisotopic labels include 3H, 111In, 125I, 32P, 35S, 14C, 57Co, 59Fe, 75Se, 152Eu, 15 90Y, 67Cu, 211At, 212Pb, 47Sc, 109Pd, 11C, 19F, 123I, etc.

Examples of suitable non-radioactive isotopic labels include 157Gd, 55Mn, 162Dy, 52Tr, 46Fe, etc.

Examples of suitable fluorescent labels include a 152Eu label, a fluorescein label, an isothiocyanate 20 label, a rhodamine label, a phycoerythrin label, a phycodyanin label, an allophycocyanin label, a fluorescamine label, etc.

Examples of chemiluminescent labels include a luminal label, an isoluminal label, an aromatic 25 acridinium ester label, an imidazole label, an acridinium salt label, an oxalate ester label, a luciferin label, a luciferase label, etc.

Those of ordinary skill in the art will know of other suitable labels which may be employed in 30 accordance with the present invention. The binding of these labels to ligands and to antibodies or fragments thereof can be accomplished using standard techniques commonly known to those of ordinary skill in the art. Typical techniques are described by Kennedy, J. H., et 35 al., 1976 (Clin. Chim. Acta 70:1-31), and Schurs, A. H.

W. M., et al. 1977 (*Clin. Chim Acta* 81:1-40). Coupling techniques mentioned in the latter are the glutaraldehyde method, the periodate method, the dimaleimide method, and others, all of which are incorporated by reference herein.

The detection of antibodies (or fragments of antibodies) of the present invention can be improved through the use of carriers. Well-known carriers include glass, polystyrene, polypropylene, 10 polyethylene, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may 15 have virtually any possible structural configuration so long as the coupled molecule is capable of binding to PAP. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface 20 of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Those skilled in the art will note many other suitable carriers for binding monoclonal antibody, or will be able to ascertain the same by use of routine experimentation.

25 The ligands or antibodies, or fragments of  
antibodies or ligands of PAPs discussed above may be  
used to quantitatively or qualitatively detect the  
presence of PAP. Such detection may be accomplished  
using any of a variety of immunoassays known to  
30 persons of ordinary skill in the art such as  
radioimmunoassays, immunometric assays, etc. Using  
standard methodology well known in the art, a  
diagnostic assay can be constructed by coating on a  
surface (i.e. a solid support) for example, a  
35 microtitration plate or a membrane (e.g.

nitrocellulose membrane), antibodies specific for PAP or a portion of PAP, and contacting it with a sample from a person suspected of having a PAP related disease. The presence of a resulting complex formed

5 between PAP in the sample and antibodies specific therefor can be detected by any of the known detection methods common in the art such as fluorescent antibody spectroscopy or colorimetry. A good description of a radioimmune assay may be found in Laboratory

10 Techniques and Biochemistry in Molecular Biology. by Work, T.S., et al. North Holland Publishing Company, N.Y. (1978), incorporated by reference herein. Sandwich assays are described by Wide at pages 199-206 of Radioimmune Assay Method, edited by Kirkham and

15 Hunter, E. & S. Livingstone, Edinburgh, 1970.

The diagnostic methods of this invention can be predictive of diseases involving PBR including gallstones, atherosclerosis, Niemann-Pick C, Sitosterolemia, Dystrophy, Tumor proliferation

20 (tumorigenesis), Schnyder's corneal crystalline dystrophy. Brain disorders include cholesterol metabolism and Alzheimer's disease, Tellurium toxicity, Smith-Lemli-Opitz syndrome, myelinization, developmental abnormalities and demyelization :

25 Charcot-Marie-Tooth disease; Pelizaeus-Merzbacher disease, Multiple sclerosis, SLA, to name a few. Alternatively, the methods and compositions may be useful as prophylactic treatment, or in screening for compounds effective in prophylactic treatments.

30 The recombinant protein can be used to identify inhibitors or activators of a PAP activity which allows the identification of drugs or agents which modulate PBR activity. Using an assay as described below in the Examples, or for example, introducing a

drug or agent to a cell expressing a PAP and detecting a increase or decrease in the level of PAP RNA or protein, natural and synthetic agents and drugs can be discovered which result in a reduction or elimination, 5 or increase in a PAP activity. Knowledge of the mechanism of action of the inhibitor or activator is not necessary as long as a decrease or increase in the activity of a PAP is detected. Inhibitors may include agents or drugs which either bind or sequester the 10 PAP's substrate(s), such as PBR, or cofactor(s), or inhibit PAP itself, directly, for example by irreversible binding of the agent or drug to the PAP, or indirectly, for example by introducing an agent which binds the competes with PAP binding to its 15 substrate. Activators may include cofactors necessary for proper PAP function or agents which allow a higher turnover rate of binding or release of the PAP to/from PBR or the particular PAP substrate. Agents or drugs related to this invention may result in partial or 20 complete inhibition or various degrees of activation of PAP which may or may not result in modulation of PBR function. Inhibitors or activators of PAP activity may be used in the treatment or amelioration of conditions such as stress, cancer, 25 neurodegenerative disorders, i.e. stroke, Alzheimer's, developmental disorders, infertility, and immune disorders.

Agents which decrease the level of PAP (i.e. in a human or an animal) or reduce or inhibit PAP activity may be used in the therapy of any disease associated with the elevated levels of PAP. Similarly, agents which increase the level of PAP or activate PAP activity may be used in the therapy of any disease associated with reduced levels of PAP. An increase or decrease in the level of PAP is determined when the

change in the level of PAP is about 2-3 fold higher or lower than the level of PAP in the normal cell, up to about 10-100 fold higher or lower than the amount of PAP in a normal cell. Agents which decrease PAP RNA

5 include, but are not limited to, one or more ribozymes capable of digesting PAP RNA, or antisense oligonucleotides capable of hybridizing to PAP RNA such that the translation of PAP is inhibited or reduced resulting in a decrease in the level of PAP.

10 These agents can be administered as DNA, as DNA entrapped in proteoliposomes containing viral envelope receptor proteins (Kanoda, Y. et al., 1989, *Science* 243, 375) or as part of a vector which can be expressed in the target cell such that the DNA or RNA

15 is made. Vectors which are expressed in particular cell types are known in the art, for example, for the mammary gland, please see Furth, (1997) (*J. Mammary Gland Biol. Neopl.* 2, 373) for examples of conditional control of gene expression in the mammary gland.

20 Alternatively, the DNA can be injected along with a carrier. A carrier can be a protein such as a cytokine, for example interleukin 2, or polylysine-glycoprotein carrier. Such carrier proteins and vectors and methods of using same are known in the

25 art. In addition, the DNA could be coated onto tiny gold beads and said beads introduced into the skin with, for example, a gene gun (Ulmer, J. B. et al., 1993, *Science* 259, 1745).

30 Alternatively, antibodies, or compounds capable of reducing or inhibiting PAP activity, that is reducing or inhibiting either the expression, production or activity of PAP, such as antagonists, can be provided as an isolated and substantially purified protein, or as part of an expression vector

35 capable of being expressed in the target cell such

that the PAP-reducing or inhibiting agent is produced. Similarly, compounds capable of increasing or activating PAP activity, that is increasing or activating either the expression, production, or 5 activity of PAP, such as agonists, can be provided as an isolated and substantially purified protein, or as part of an expression vector capable of being expressed in the target cell such that the PAP-elevating or activating agent is produced. In 10 addition, factors which affect the stability of the protein, and co-factors such as various ions, i.e. Ca<sup>2+</sup> (Calvo, D. J. and Medina, J. H., 1993, *J. Recept. Res.* 13:975-987), or anions, such as halides or anion channel blockers such as DIDS 15 (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid), an ion transport blocker (Skolnick, P., 1987, *Eur. J. Pharmacol.* 133:205-214), or factors which affect the stability of PBR such as lipids, for example, the phospholipids phosphatidylserine and 20 phosphatidylinositol whereby the presence of the phospholipids is required for receptor activity (Moynagh, P. N. and Williams, D.C., 1992, *Biochem. Pharmacol.* 43:1939-1945) can be administered to modulate the expression and function of the PAPs and 25 PBR. These formulations can be administered by standard routes. In general, the combinations may be administered by the topical, transdermal, intraperitoneal, oral, rectal, or parenteral (e.g. intravenous, subcutaneous, or intramuscular) route. 30 In addition, PAP-inhibiting or PAP-activating compounds may be incorporated into biodegradable polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of a tumor or implanted so that the PAP-inhibiting or PAP- 35 activating compound is slowly released systemically.

The biodegradable polymers and their use are described, for example, in detail in Brem et al. (1991) *J. Neurosurg.* 74, 441-446. These compounds are intended to be provided to recipient subjects in an 5 amount sufficient to effect the inhibition of PAP. Similarly, agents which are capable of negatively or positively effecting the expression, production, stability or function of PAP, are intended to be provided to recipient subjects in an amount sufficient 10 to result in the desired effect. An amount is said to be sufficient to "effect" the inhibition or induction of PAP if the dosage, route of administration, etc. of the agent are sufficient to influence such a response.

The PAPs identified in this application were 15 discovered due to their ability to associate with PBR, and may play a role in the proper targeting, function, expression, or stability of PBR. Therefore, a method for inhibiting or reducing PBR function, or altering the localization of PBR, would include a method for 20 dissociating PAPs from the receptor. This is possible using agents which block the site on PBR at which these PAPs associate with PBR, or alternatively, blocking the site on the PAPs which is involved in PBR-association. Such agents would include antibodies 25 or antagonists which recognize such sites or which alter the conformation of these sites such that PAP and PBR association is inhibited or eliminated. Agents which decrease the level of PBR (i.e. in a human or an animal) or reduce or inhibit PBR activity 30 may be used in the therapy of any disease associated with the elevated levels of PBR such as metastatic cancer, for example breast cancer, or diseases associated with increased cell proliferation or increased cholesterol transport into the cell. An 35 increase in the level of PBR is determined when the

level of PBR in a tumor cell is about 2-3 times the level of PBR in the normal cell, up to about 10-100 times the amount of PBR in a normal cell.

Antibodies or compounds capable of reducing or 5 inhibiting the association between PBR and PAPs can be provided as an isolated and substantially purified protein, or as part of an expression vector capable of being expressed in the target cell such that the PBR-PAP-association reducing or inhibiting agent is 10 produced. These formulations can be administered by standard routes. In general, the combinations may be administered by the topical, transdermal, intraperitoneal, oral, rectal, or parenteral (e.g. intravenous, subcutaneous, or intramuscular) route. 15 In addition, these compounds may be incorporated into biodegradable polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of a tumor or implanted so that the compound is slowly released systemically. The biodegradable 20 polymers and their use are described, for example, in detail in Brem et al. (1991) *J. Neurosurg.* 74, 441-446.

These compounds are intended to be provided to recipient subjects in an amount sufficient to effect the inhibition of PBR/PAP association.

25 In line with the function of PBR in cell proliferation, agents which stimulate the function of PBR by increasing the association of PAPs to PBR, may be used in the therapy of any disease associated with a decrease of PBR, or a decrease in cell 30 proliferation, wherein PBR is capable of increasing such proliferation, e.g. developmental retardation. PBR has also been shown to be involved in cholesterol transport, therefore, an agent or drug which results in an increase in function or stability of PBR and its 35 associated PAPs can be used to increase cholesterol

transport into cells. Diseases where cholesterol transport is deficient include lipoidal adrenal hyperplasia, and diseases where there is a requirement for increased production of compounds requiring cholesterol such as myelin and myelination including Alzheimer's disease, spinal chord injury, and brain development neuropathy [Snipes, G. and Suter, U. (1997) Cholesterol and Myelin. In: Subcellular Biochemistry, Robert Bittman (ed.), vol. 28, pp.173-204, Plenum Press, New York], to name a few.

In providing a patient with any agent which modulates the expression, function, targeting, or association of PAP or PBR as discussed above, the dosage of administered agent will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of agent which is in the range of from about 1 pg/kg to 10 mg/kg (body weight of patient), although a lower or higher dosage may be administered.

A composition is said to be "pharmacologically acceptable" if its administration can be tolerated by a recipient patient. Such an agent is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient.

The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby these materials, or their functional derivatives, are combined in admixture with a pharmaceutically

acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in Remington's Pharmaceutical Sciences [16th ed., 5 Osol, A. ed., Mack Easton PA. (1980)]. In order to, form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the above-described compounds together with a suitable 10 amount of carrier vehicle.

Additional pharmaceutical methods may be employed to control the duration of action. Control release preparations may be achieved through the use of polymers to complex or absorb the compounds. The 15 controlled delivery may be exercised by selecting appropriate macromolecules (for example polyesters, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate) and the 20 concentration of macromolecules as well as the method of incorporation in order to control release. Another possible method to control the duration of action by controlled release preparations is to incorporate the compounds of the present invention into particles of a 25 polymeric material such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, instead of incorporating these agents into polymeric particles, it is possible to entrap these materials in 30 microcapsules prepared, for example, interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly(methylmethacrylate) microcapsules, respectively, or in colloidal drug delivery systems, for example, 35 liposomes, albumin microspheres, microemulsions,

nanoparticles, and nanocapsules or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences (1980).

The present invention also provides kits for use 5 in the diagnostic or therapeutic methods described above. Kits according to this aspect of the invention may comprise one or more containers, such as vials, tubes, ampules, bottles and the like, which may comprise one or more of the compositions of the 10 invention.

The kits of the invention may comprise one or more of the following components, one or more compounds or compositions of the invention, and one or more excipient, diluent, or adjuvant.

15 It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are obvious and may be made without departing from the scope of the invention 20 or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting 25 of the invention.

The following Materials and Methods were used in the Examples described below.

#### **Material and Methods**

##### Materials

30  $[\alpha-^{32}\text{P}]$ dCTP (specific actifity, 3000 Ci/mmol),  $[1,2,6,7-\text{N-}^3\text{H}]$ progesterone (specific activity, 94.1 Ci/mmol) and  $^3\text{H-1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinolinecarboxamide}$  (PK 11195) (specific activity, 86.9 Ci/mmol) were obtained from

NEN Life Science Products (Boston, MA). PK11195 was obtained from Research Biochemicals, Inc. (Natick, MA). Nitrocellulose (0.45  $\mu$ m) was from Hoeffer Scientific (San Francisco, CA). 22R

5 Hydroxycholesterol was purchased from Sigma. Restriction enzymes were from Stratagene (La Jolla, CA) and New England Biolabs (Beverly, MA). Cell culture supplies were purchased from from Life Technologies, Inc. (Grand Island, NY). Tissue culture

10 plasticware was from Corning (Corning, NY). Electrophoresis reagents and materials were supplied from BioRad. All other chemicals used were of analytical grade and were obtained from various commercial sources.

15 Strains and media

The genotype of the *Saccharomyces cerevisiae* reporter strain HF7c is *MATA*, *ura3-52*, *his3-200*, *lys2-801*, *ade2-101*, *trp1-901*, *leu2-3, 112*, *gal4-542*, *gal80-538*, *LYS2::GAL-HIS3*, *URA3::(GAL4 17-mers),-CYC1-lacZ*

20 (CLONTECH, Palo Alto, CA). Yeast strains were grown at 30°C in standard liquid YPD medium or minimal SD synthetic medium with appropriate supplement amino acids (CLONTECH, Palo Alto, CA).

25 Plasmids and construction

The mouse PBR cDNA coding sequence was subcloned into pGBT9 (CLONTECH, Palo Alto, CA) at *EcoR I* and *BamH I* sites (pGBT-PBR). The fusion site was verified by sequencing. Functional fusion PBR protein, expressed in yeast cells, was verified by PBR ligand binding assay. Mouse testis cDNA library was constructed in pGAD10 [LEU2, GAL4 (768-881)] (CLONTECH, Palo Alto, CA). Amplification of premade libraries was performed by growing the transformants on LB-agar-ampicillin and purifying the plasmids DNA

with QIAGEN Plasmid Giga kit (QIAGEN, Valencia, CA). In the transfection experiments, PAP7 partial sequence (including 192 Amino acids C-terminal sequence) was inserted into pSVzeo vector (Invitrogen, Carlsbad, CA) at *EcoRI* and *BamH1* sites.

Yeast two-hybrid screening

The Clontech MATCHMAKER two-hybrid system was applied in this study (detailed in manufacturer's instruction book). Briefly, the yeast reporter host strain HF7c was simultaneously cotransformed with both pGBT-PBR and the mouse testis cDNA library in pGAD10 plasmid by using lithium acetate high-efficiency method (Gietz, D. et al., 1992, *Nucleic Acids Res.* 20, 1425). HIS positive clones were further selected by colony lift filter assay for  $\beta$ -galactosidase activity. Plasmid DNA was rescued in *Escherichia coli* DH5 $\alpha$  from yeast cells. Plasmids were retransformed into yeast HF7c cells with plasmid pGBT-PBR to test for histidine prototrophy and  $\beta$ -galactosidase activity (Clontech manual). The cDNA inserts from the positive clones were sequenced. The full length PAP7 cDNA was obtained by using 5' and 3' RACE kit from kit (CLONTECH, Palo Alto, CA).

Sequence analysis

The ABI PRISM<sup>TM</sup> dyes terminator cycle sequencing ready reaction kit (PE Biosystems, Foster City, CA) and an Applied Biosystems sequencer were used for sequencing (Applied Biosystems, Foster City, CA) at the Lombardi Cancer Center Sequencing Core Facility (Georgetown University). DNA sequences were analyzed by using Entrez and BLAST program against GeneBank<sup>TM</sup> Database.

Cell culture transient transfection

MA-10 cells were grown in modified Waymouth's MB752/1 medium containing 15% horse serum, as described previously (Papadopoulos, V. et al., 1990, 5 *J. Biol. Chem.* 265, 3772-3779). Mouse C6 glioma and mouse Y1 adrenal cortical cells were cultured in DMEM and DMEM F12 repectively, with 10% fetal bovine serum. MA10 cells were transiently transfected by electroporation (El Hefnawy, T. et al., 1996, *Mol. 10 Cell Endocrinol.* 119, 207-217). Each Genepulser cuvette (0.4 cm-gap, BioRad, Hercules, CA) contained 8x10<sup>6</sup> cells in 350 µl antibiotic-free complete Waymouth's growth medium (see above), plus 30µg plasmids DNA in 50 µl of 0.1 x TE. Cells in 15 electroporation cuvettes were electro-shocked at 330 V and at a capacitance of 950 µFd generated from Genepulser (BioRad, Hercules, CA). The cells were kept immediately on ice for 10 min before plated into 96 well plates.

20 Radioligand binding assays

<sup>3</sup>H-1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinolinecarboxamide (PK 11195) (NEN, Boston, MA) binding studies were performed as we previously described (Papadopoulos, V. et al., 1990, 25 *supra*; Garnier, M. et al., 1994, *Molecular Pharmacology* 45, 201-211). The dissociation constant (Kd) and the number of binding sites (Bmax) were determined by Scatchard plot analysis of the data using the LIGAND program (Munson, P. J. and Rodbard, 30 D., 1980, *Anal. Biochem.* 107, 220-239).

RNA (Northern) blot analysis

Total tissue and cellular RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method using RNA STAT60 reagent (Tel-Test

Inc., Friendswood, TX). RNA was separated by denature electrophoresis and transferred to Nytran membrane (Schleicher & Schuell Inc., Keene, NH). The RNA blots were hybridized with  $^{32}\text{P}$  labeled PAP7 cDNA probe 5 generated from random priming (Boehringer Mannheim, Indianapolis, IN). Autoradiography was performed by exposing Kodak X-Omat AR films (Eastman Kodak, Rochester, NY) to the blots at  $-80^{\circ}\text{C}$  overnight.

Steroid biosynthesis

10 MA-10 cells were plated into 96-well plate at the density of  $2.5 \times 10^4$ /well for overnight. The cells were stimulated with 50 ng/ml hCG in 0.2 ml/well serum-free medium for 2 hours. The culture medium was collected and tested for progesterone production by RIA. The 15 assay was carried out by using anti-progesterone antisera (ICN, Costa Mesa, CA), following the conditions recommended by the manufacturer. The progesterone production was normalized by the amount of protein in each well. Radioimmunoassay data was 20 analyzed using the software provided by Wallac (EG&G Wallac, Gaithersburg, MD).

Antibody generation and Western analysis

Rabbit anti-PAP7 antibody was prepared by sequential immunization with a peptide 25 SSDEEEEEENVTCEEKAKKNKP (SEQ ID NO:11) of PAP7 protein, which was coupled to KLH. PAP7 antibodies were purified by an affinity resin containing the same peptide immobilized onto agarose (Bethyl Laboratories, Montgomery, TX). MA10 cells were solubilized in 30 sample buffer (25 mM Tris-HCl (pH6.8), 1% SDS, 5%  $\beta$ -mercaptoethanol, 1 mM EDTA, 4% glycerol, and 0.01% bromophenol blue), boiled for 5 min, and loaded onto a 15% SDS-PAGE minigel (MiniProtein II System, BioRad, Richmond, CA). Electrophoresis was performed at 25

mA/gel using a standard SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS). The proteins were electrophoretically transferred to nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH). The 5 membrane was incubated in blocking buffer (TTBS buffer (20 mM Tris/HCl, pH 7.5, 0.5M NaCl, and 0.05% Tween-20) containing 10% Carnation nonfat milk) at room temperature for 1 hour, followed by incubation with a primary antibody against PAP7 (1:2000) for 2 hours. 10 The membrane was washed with TTBS three times for 10 min each time. After one-hour incubation with the secondary antibody, goat anti-rabbit IgG conjugated with HRP (Signal Transduction), the membrane was washed with TTBS three times for 10 min each time. 15 Specific protein bands were detected by chemiluminescence using the Renaissance Kit (DuPont-New England Nuclear, Wilmington, DE) according to manufacturer's directions.

Immunocytochemistry

20 MA-10 cells were cultured on four-chambered SuperCell Culture Slides (Fisher Scientific, Pittsburgh, PA) and fixed with methanol at 4°C for 15 min. The fixed cells were incubated with PAP7 antibody (1:250 dilution) with or without PAP7 peptide 25 for 1 hour. After washing, the cells were incubated with HRP conjugated goat anti-rabbit secondary antibody (Transduction Lab, Lexington, KY) for 1 hour. PAP7 staining was visualized with peroxidase using AEC (3-amino-9-ethyl carbazole) as a chromogen to yield a 30 red reaction product. After counterstaining with hematoxylin, slides were dehydrated and permanently mounted.

Immunohistochemistry

Mouse tissues were freshly snipped in liquid nitrogen. Specimens were fixed in cold methanol right after sectioning for 5 min. The slides were then placed in a chamber containing 0.3% H<sub>2</sub>O<sub>2</sub> solution in 5 methanol for 20 min at room temperature to inhibit the endogenous peroxidase activity and then incubated in blocking solution (10% goat serum) (Zymed, South San Francisco, CA) for 15 min. Subsequently, the slides were incubated with anti-PAP7 antibody (1:250) for 2 h 10 at room temperature, washed with water and PBS, incubated with HRP conjugated goat anti-rabbit secondary antibody for 1 hour at room temperature, and then washed with PBS. After treatment with AEC reagent for 1 hr at 37°C for color staining, the 15 sections were counterstained with hematoxylin, dehydrated and permanently mounted.

Protein quantification and Statistical analysis

Proteins were quantified by dye-binding assay of Bradford (Bradford, M. M., 1976, *Anal. Biochem.* 72, 20 248-254) with bovine serum albumin as the standard. Statistical analysis was performed by ANOVA followed by the Student-Newman-Keuls test or the Dunnett multiple comparisons test using the Instat (v.2.04) package from GraphPad, Inc. (San Diego, CA).

25 Example 1

Isolation of PBR Associated Proteins

We have used the MATCHMAKER Two-Hybrid System from CLONTECH in order to clone genes whose products interact with PBR protein. GAL4 (1-147)-PBR fusion 30 (plasmid pGBT9 + PBR) was used as a bait to screen a mouse MATCHMAKER testis cDNA library constructed into the pGAD10 two-hybrid vector. About 3x10<sup>6</sup> transformants were tested, and five positive clones were obtained for their ability to interact with PBR.

Library plasmids from these transformants were rescued in *E. coli* strain DH5 $\alpha$ . Both the His<sup>+</sup> phenotype and the expression of  $\beta$ -galactosidase were confirmed by a second-round transformation of strain HF7c carrying 5 pGBT9-PBR (Table 1).

Table 1: Summary of yeast two hybrid screen of mouse testis library by PBR

Clone	His3	$\beta$ -Galactosidase
activity		
PAP3	+	++
PAP7	+	++
PAP20	+	++
Positive control	+	+++

15 Plasmids from these positive clones were first analyzed by restriction enzyme digestion and followed by sequence analysis. Two clones were shown to be coded by single gene, which was an unknown gene and 20 was named as PBR associated protein 7 (PAP7). The other three clones encoded different products. After 5' RACE and 3' RACE, PAP7 cDNA clone was completely sequenced on both strands (SEQ ID NO:2) and it encoded a 463-amino acid protein with a calculated molecular 25 weight of about 52 kDa. A homology search in the Genebank database using the BLAST program showed that this is a new sequence, previously unidentified.

Example 2

PAP7 protein expression in MA-10 Leydig tumor 30 cells

The total MA-10 cell protein extracts were analyzed by western blot using PAP7 antibody. This antibody specifically recognizes a 50 kDa-protein band (Fig. 1A). The PAP7 protein expression in MA-10 cell

was also checked by immunocytochemistry. PAP7 antibody specifically stained MA-10 cell, with the signal mostly localized in the cytoplasm (Figure 1B). This signal can be neutralized by PAP7 peptide, which 5 was used to generate and purify this antibody.

Example 3

PAP7 cells and tissue expression by Dot and Northern Blot

By dot blot analysis, PAP7 was observed to be 10 highly expressed in brain, eye, submax gland, testis, and ovary. Interestingly, PAP7 expression was at its highest level at early embryonic stage, and decreased before birth (Fig. 2A and 2B). Consistently, PAP7 mRNA was expressed in adrenal, brain, heart, liver, testis 15 and ovarian tissues by Northern blot analysis. PAP7 had a 1 kb transcript which was only expressed in testis and a 3 Kb major mRNA transcript in the other tissues (Fig. 3A and 3B). PAP7 was also highly expressed in three cell lines, C6 glioma, MA-10 Leydig 20 cells and Y1 adrenal cells, which have been widely used for studying steroid biosynthesis. All three cell lines expressed PAP7 transcript of the same molecular weight size as in normal tissues. The PAP7 expression level in these cell lines was 25 proportionally correlated with their steroidogenic capability (Fig. 3A and 3B). The PBR mRNA expression level was also checked in these same tissues and cell lines. The PBR expression level was parallel with PAP7 mRNA expression pattern, especially in those 30 three cell lines (Fig. 4A and 4B).

Example 4

PAP7 Cellular distribution

PAP7 protein expression in different tissues was checked by immunohistochemistry (Fig 5). PAP7 was

present in both Leydig and germ cells in testis (Figure 5C and 5D), in hippocampus and neuronal cells in brain (Figure 5E and 5F), in fasciculata cells in adrenal gland (Figure 5G and 5H), and in granulosa 5 cells in ovary (Figure 5A and 5B). Liver and kidney expressed low level of PAP7 protein (data not shown). Each specimen was stained with PAP7 peptide neutralized antibody as a negative control. Subsequent in situ hybridization studies showed that 10 PAP7 mRNA followed PAP-7 protein expression.

Example 5

The effect of PAP7 on steroid biosynthesis in MA-10 cell

PAP7 partial sequence including PBR binding 15 domain was subcloned into pSVzeo mammalian expression vector. This pSVzeoPAP7 vector was transiently transfected into MA-10 cells. pSVzeo empty vector was also transfected into cells as control. The capability of steroid biosynthesis of both empty 20 vector pSVzeo transfectants and pSVzeoPAP7 transfectants was checked by monitoring the progesterone production in response to hormonal (hCG) stimulation. PAP7 transfectants had significantly reduced the level of progesterone production in MA-10 25 cells as compared with pSVzeo vector transfectant at a dose and time dependent manner (Fig 6).

Discussion

In order to better understand the mechanism of 30 how PBR regulates cholesterol transport activity in steroid biosynthesis, we performed yeast two-hybrid assay to identify the PBR associated protein(s). Mouse PBR cDNA was inserted into the pGBT9 vector to generate a GAL4 DNA-binding domain and PBR fusion

protein as bait. The receptor ligand binding activity of the fusion protein with PBR ligand, PK11195, was tested. Our result indicated that the fusion PBR protein expressed in yeast possessed the similar binding affinity as the native PBR protein (data not shown). PBR has been identified in various peripheral tissues (Gavish, M. and Weizman, R., 1997, *Clin. Neuropharmacol.* 20, 473-481) including testis. Testis is one of the important and very well studied tissues for steroidogenesis (Huhtaniemi, I., and Toppari, J., 1995, *Adv. Exp. Med. Biol.* 377, 33-54). The steroid biosynthesis in the mouse testicular Leydig cell and the role of PBR in this process are also well documented (Papadopoulos, V. et al., 1997, *Steroids* 62, 21-28; Papadopoulos, V. et al., 1998, *Endocr. Res.* 24, 479-487). Therefore, we applied mouse testis cDNA library in this two-hybrid screen study. The pGAD10 vector was used to generate a fusion protein of the GAL4 activation domain with a collection of random proteins in the fusion Balb/c mouse testis library. Through the yeast two hybrid screen, PAP7 was identified as one of the positive clones, which demonstrated its ability to interact with PBR (Table 1). Thus, we cloned the PAP7 cDNA coding for a mouse protein that interacted with PBR. Based on the database search, PAP7 is a novel gene product. Recently, PRAX-1 was reported as a new protein that specifically interates with PBR (Gallegue, S. et al., 1999, *J. Biol. Chem.* 274, 2938-2952). The only similarity is that both proteins contain glutamic-acid stretches. Part of PAP7 shares quite high homology with a *C. elegans* gene that has an unknown function (Wilson, R. et al., 1994, *Nature* 368, 32-38). In fact, cholesterol is required for *C. elegans* cell culture (Brenner, S., 1974, *Genetics* 77, 71-94).

Considering that PBR is involved in cholesterol transport and PBR gene is highly conservative in all type of organisms, this data suggests that PAP7 expression may be needed to meet basic requirements 5 for cell survival and growth. PAP7 also shares some homologies with RALBP, a hydrophobic ligand-binding protein that functions in intracellular retinoid transport (Ozaki, K. et al., 1994, *J. Biol. Chem.* 269, 3838-3845).

10 By sequence motif analysis using Swiss-Port Prosite profile scan, PAP7 has fatty acylation (myristoylation) sites, Acyl-CoA-binding protein signature and PKC phosphorylation sites. Protein myristoylation enables protein to attach to the 15 cellular membrane and thus take part in cell signaling (Casey, P.J., 1995, *Science* 268, 221-225; Boutin, J. A., 1997, *Cell Signal* 9, 15-35). PBR is a hydrophobic protein and tightly associated with the outer mitochondrial membrane. This property could enable 20 PAP7 passing hormone stimulation signal to and interacting with PBR thus regulating PBR activity in cholesterol transport. Interestingly, Acyl-CoA-binding protein is the other name of PBR endogenous ligand, diazepam binding inhibitor (DBI) (Rose, T. M. 25 et al., 1992, *Proc. Natl. Acad. Sci. U.S.A.* 89, 11287-11291; Costa, E. and Guidotti, A., 1991, *Life Sci.* 49, 325-344; Suk, K. et al., 1999, *Biochim. Biophys. Acta* 1454, 126-131). This information suggests that PAP7 may coordinate with other PBR endogenous ligands to 30 fulfill its function. The fact that PAP7 has the potential protein kinase phosphorylation sites raises another possibility that PBR could be regulated by hormone stimulation through the interaction with PAP7 protein.

The distribution and expression of PAP7 were examined in major mouse tissues such as brain, testis, ovary, adrenal, and kidney, as well as some cell lines. The PAP7 expressing pattern is similar to the 5 broader expression profile of PBR. According to the previous studies (Papadopoulos, V. et al., 1998, *supra*), glucocorticoids are produced by zona fasciculata cells in adrenal. In the ovary, corpus luteum, where the granulosa cells are located, 10 secretes progesterone. In addition, the testicular Leydig cells are able to produce testosterone. Since PAP7 is highly expressed in major steroidogenic tissues and is more concentrated in these steroid producing cells, PAP7 may be involved in steroid 15 biosynthesis or the regulation of steroid formation by changing the formation or the conformation of the PBR complex. Mouse C6 glioma cells, MA-10 Leydig cells and Y1 adrenal cortical cells are popular cell models selected for studying steroid biosynthesis. PAP7 20 expression is proportionally correlated to the PBR expression in these cell lines. Additionally, both PBR and PAP7 expression level in these cell lines are parallel with their steroidogenic capability, which also suggests that PAP7 may be involved in steroid 25 biosynthesis through a PBR pathway. A small PAP7 transcript was expressed only in testis, a phenomenon observed for other genes expressed in testis (Zhang, F. P. et al., 1997, *Endocrinology* 138, 2481-2490; Mauduit, C. et al., 1999, *J. Biol. Chem.* 274, 770- 30 775). The immunostaining in the testis other than in Leydig cells could represent the expression of the smaller transcript.

The PAP7 protein is expressed in MA-10 cells and most of the staining is localized in the cytoplasm. 35 The study of PAP7 subcellular distribution is ongoing.

the results of which may provide more detailed information about the interaction between PBR and PAP7. Since PBR knockout mice die in uterus indicates an essential role for PBR essential in mouse embryonic 5 development. Interestingly, PAP7 mRNA is highly expressed during mouse early embryonic development. This result may suggest that PAP7, associated with PBR, could play an important role during early mouse development. This further implies that PBR may have 10 new functions beyond steroidogenesis. Overexpression of PAP7 fragment including its PBR binding domain significantly inhibited the progesterone formation stimulated by saturating concentrations of hCG (50 ng/ml) in MA-10 cells. According to previous studies, 15 progesterone production of these cells represented the index of steroid biosynthesis (Freeman, D. A., 1987, *Endocrinology* 120, 124-132; Garnier, M. et al., 1994, *J. Biol. Chem.* 269, 22105-22112). Based on the inhibitory manner, we assume that the overexpressed 20 PAP7 fragment might act as a competitor of the native PAP7 in MA-10 cells and competitively bind to PBR. We believe that the tranfected PAP7 fragment having only the PBR binding domain and is not fully functional as a native PAP7, however, it competitively prevents PBR 25 from interacting with the endogenous PAP7 and thereby blocks the normal function of PBR.

In conclusion, the results presented herein suggest that the identified PAP7 is involved in the regulation of the PBR function, serving as an 30 endogenous ligand or allosteric modulator of the receptor.

Considering the findings tha i) PBR is a channel/transporter of cholesterol, ii) PBR is the target of environmental antisteroidogenic hazards, and 35 (iii) PBR is involved in breast cancer aggression and

tumor cell proliferation (Hardwick, M. et al., 1999,  
Cancer Res. 59, 831-842), we believe that the  
identification and characterization of PAP7 will  
greatly contribute to the understanding of the role of  
5 PBR in steroidogenesis and even in more general areas.

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SEQ ID NO:1

PAP3 nucleic acid

GAATTCGGCCCGCTGACCCGCTGCGCCCTCTCGAGGCCGCTGAACGAGGAATA  
 ATTGCTAATAAGGCCTCTGTAGCCATGGCTACTCTGACGTAAACCAAAATCAATAAGTCGTG  
 CCAAGAAATGGTCAGAGGAATAGAAAATCTGTACAGATTCAACAAGCAGGATATCGGGATG  
 AAATTGAATATAAACAAAGTGAACAAAGTGGCCATGGTCGACCGATGGCCAGAGACAGGGTACG  
 TGAAGAAACTTCAGCGGAGGGACAATACTTCTACTACAAACAAGAGAGGGAGTGCAG  
 GGACAAGGAGGTCCACAAAGTGAAGGTTACGCTACTGACCTTTCTCGGCTTG  
 GCAATGCTCCTTAAGAATTGGTTGTTACATTCTCATCGTAAATGTCATTTACAA  
 AACAAATTACAATTCTGTCTTAATTCATGGTGTCTTACACAAACATAAACACCCACCTTGA  
 AACCCAAAAA

SEQ ID NO:2

PAP7 sequence:

GAATTCGGCCCGCTGACCTAAAGTTGAGTTCACTGTAGTGACCCGTGTGAAGGTAGTT  
 TTATTTTAAATCAACTTTCATTGTGCAAACACTAGTAAAGATGGCAAAGCCTTCATCCAACCTA  
 TGAAGAAAACACTGAAGTTCGTGGCACTGCATAAGCAAGTTCTTGGGCCATATAACCCAGA  
 CACGTCCCTGAGGTTGGATTCTTGATGTGTTGGGAATGATAGGAGGAGAGAATGGCAGCT  
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 TTAATTCTTT

SEQ ID NO:3

PAP8 nucleic acid

GAATTCGGCCCGCTGACACAAGCGTGGAGCGAAGAGCCCTGGAGAGATTCA  
 AATGTAGGTGAAGGCTCTTCAACATCACAGGGCACCTGGCAGTCTCAGAGTCCTCACAGTCA  
 AACCTGGGGAGCAGACGCAAGCGGACCCAGGGAGGAAGGTGTCAAGCTGGAGAGGCA  
 TAACCGAATGGAACGAGATAGGAGGCCAGAATCCGATTGCTGTGATGAGCTGAATCTTTA  
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ACCGGCCGTTCTGCAGCATGCACATGCCTGCCATCCCCGGAATTCTAGCTCTGAATCCT  
CTC

SEQ ID NO: 4

### PAP15 nucleic acid

GAATTCCGGCCGCGTCGACCTCGGGGTAACCTTGGGATCATTATGATGTCACCTCTCAATCTCTGAGACCAGTATTATGAACTGACATTGGTGCTGGTGGGCTTGGAAAGCAGAGGCCACCTTGTCTGTAGAGGATACTGAGCGGCTGGATGGCAGGAATCCAAGAGAAGCCAGGCTCTCGTAGTCGCCCCGGACAGTGGAGAGAGGGAGGACCGGAAAGGGATCTGTTCTCCTAGGCCGAGCATGTCCTGTGGACATGCTCTGTGACAGCTGAGCCTGCCAGCCTGCCCTCTGAAGTGGGTGTCCTGCCACAACCAAGCCAGCAATCGGTCTGTTTCCGACAACCTCAGAGCCAGACCTACAAGCCTATTGGTGGTCCAAAATTCTCTCAGATCTCATGTCATCCCTCCACTCCCTCCAAAAGAGAAAGAAATTGAGAAAGAA

SEQ ID NO:5

### PAP20 nucleic acid

GAATTCCGGCCCGCTGACCTCAGAAGAGGAAAAGAGGTGCAAGAAGTGTGGCGGCACAAA  
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CTGATCTGGCATTAAAGTTCTGCTGCTCTATACCATCTTGTAAAGCCCAGGAACCTTCCCTG  
CGTCTCTGATTGCCACGAAACAAATGTCTTACTCACTAAAGAAAATCCATTGCTTGGTGCCT  
CTGACCCATGAATATCATAAGGG

SEQ ID NO: 6

### PAP3 amino acid sequence

```

Met Ala Thr Ser Asp Val Lys Pro Lys Ser Ile Ser Arg Ala Lys
Lys Trp Ser Glu Glu Ile Glu Asn Leu Tyr Arg Phe Gln Gln Ala
Gly Tyr Arg Asp Glu Ile Glu Tyr Lys Gln Val Lys Gln Val Ala
Met Val Asp Arg Trp Pro Glu Thr Gly Tyr Val Lys Lys Leu Gln
Arg Arg Asp Asn Thr Phe Phe Tyr Tyr Asn Lys Glu Arg Glu Cys
Glu Asp Lys Glu Val His Lys Val Lys Val Tyr Val Tyr

```

SEQ ID NO:7

### PAP7 amino acid sequence

Arg	Pro	Arg	Arg	Pro	Lys	Val	Glu	Leu	Phe	Thr	Val	Val	Thr	Arg
Val	Lys	Val	Val	Leu	Phe	Leu	Asn	Gln	Leu	Ser	Leu	Cys	Lys	Leu
Val	Lys	Asp	Gly	Lys	Ala	Phe	His	Pro	Thr	Tyr	Glu	Glu	Lys	Leu
Lys	Phe	Val	Ala	Leu	His	Lys	Gln	Val	Leu	Leu	Gly	Pro	Tyr	Asn
Pro	Asp	Thr	Ser	Pro	Glu	Val	Gly	Phe	Phe	Asp	Val	Leu	Gly	Asn
Asp	Arg	Arg	Arg	Glu	Trp	Ala	Ala	Leu	Gly	Asn	Met	Ser	Lys	Glu
Asp	Ala	Met	Val	Glu	Phe	Val	Lys	Leu	Leu	Asn	Lys	Cys	Cys	Pro

Leu Leu Ser Ala Tyr Val Ala Ser His Arg Ile Glu Lys Glu Glu  
 Glu Glu Lys Arg Arg Lys Ala Glu Glu Glu Arg Arg Gln Arg Glu  
 Glu Glu Glu Arg Glu Arg Leu Gln Lys Glu Glu Lys Arg Lys  
 Arg Glu Glu Glu Asp Arg Leu Arg Arg Glu Glu Glu Arg Arg  
 Arg Ile Glu Glu Glu Arg Leu Arg Leu Glu Gln Gln Lys Gln Gln  
 Ile Met Ala Ala Leu Asn Ser Gln Thr Ala Val Gln Phe Gln Gln  
 Tyr Ala Ala Gln Gln Tyr Pro Gly Asn Tyr Glu Gln Gln Gln Ile  
 Leu Ile Arg Gln Leu Gln Glu Gln His Tyr Gln Gln Tyr Lys His  
 Gln Ala Glu Gln Thr Gln Pro Ala Gln Gln Ala Ala Leu Gln  
 Lys Gln Gln Glu Val Val Met Ala Gly Ala Ser Leu Pro Ala Ser  
 Ser Lys Val Asn Thr Ala Gly Ala Ser Asp Thr Leu Ser Val Asn  
 Gly Gln Ala Lys Thr His Thr Glu Asn Ser Glu Lys Val Leu Glu  
 Pro Glu Ala Ala Glu Glu Ala Leu Glu Asn Gly Pro Lys Asp Ser  
 Leu Pro Val Ile Ala Ala Pro Ser Met Trp Thr Arg Pro Gln Ile  
 Lys Asp Phe Lys Glu Lys Ile Arg Gln Asp Ala Asp Ser Val Ile  
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 Asp Ile Gly Phe Gly Val Tyr Phe Glu Trp Thr Asp Ser Pro Asn  
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 Glu Glu Glu Glu Asn Val Thr Cys Glu Glu Lys Ala Lys Lys Asn  
 Ala Asn Lys Pro Leu Leu Asp Glu Ile Val Pro Val Tyr Arg Arg  
 Asp Cys His Glu Glu Val Tyr Ala Gly Ser His Gln Tyr Pro Gly  
 Arg Gly Val Tyr Leu Leu Lys Phe Asp Asn Ser Tyr Ser Leu Trp  
 Arg Ser Lys Ser Val Tyr Tyr Arg Val Tyr Tyr Thr Arg

## SEQ ID NO:8

## PAP8 amino acid sequence

Glu Phe Ala Ala Ala Ser Thr Leu Asp Thr Ser Val Glu Arg Arg  
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 Glu Gln Thr Gln Ser Gly Pro Gln Gly Gly Arg Cys Gln Arg Arg  
 Glu Arg His Asn Arg Met Glu Arg Asp Arg Arg Arg Arg Ile Arg  
 Ile Cys Cys Asp Glu Leu Asn Leu Leu Val Pro Phe Cys Asn Ala  
 Glu Thr Asp Lys Ala Thr Thr Leu Gln Trp Thr Thr Ala Phe Leu  
 Lys Tyr Ile Gln Glu Arg His Gly Asp Ser Leu Lys Lys Glu Phe  
 Glu Ser Val Phe Cys Gly Lys Thr Gly Arg Arg Leu Lys Leu Thr  
 Arg Pro Glu Ser Leu Val Thr Cys Pro Ala Gln Gly Ser Leu Gln  
 Ser Ser Pro Ala Met Glu Ile Lys

## SEQ ID NO:9

## PAP15 amino acid sequence

Ala Ala Gly Trp Gln Glu Ser Lys Glu Lys Pro Ala Ser Arg Ser  
 Arg Pro Gly Thr Val Glu Glu Arg Glu Asp Arg Gln Arg Gly Ile  
 Cys Leu Ser Pro Arg Pro Glu His Val Pro Cys Gly Thr Cys Ser  
 Val Thr Ala Glu Pro Ala Gln Pro Ala Phe Leu Lys Leu Gly Val  
 Ser Cys Pro Gln Pro Ser Gln Gln Ser Val Cys Phe Pro Thr Thr  
 Ser Glu Pro Asp Leu Thr Ser Leu Phe Trp Trp Phe Pro Lys Phe  
 Leu Ser Asp Leu His Val Tyr Pro Ser Thr Pro Ser Lys Arg Glu  
 Arg Lys Glu Leu Arg Lys Lys

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SEQ ID NO:10

PAP20 amino acid sequence

Asn Ser Arg Pro Arg Arg Pro Gln Lys Arg Lys Arg Gly Ala Glu Val Leu Ala Ala Gln Ile Val Gln Lys Thr Arg Leu Glu Arg Lys Lys Gln Glu Ala Ser Val Ser Lys Asp Ala Pro Val Pro Thr Asn Thr Lys Arg Ala Lys Lys Gln Glu Lys Ser Pro Gly Arg Ile Ala Ser Gln Ser Lys Pro Pro Met Lys Lys Ser Pro Gln Lys Arg Lys Val Asn Val Ala Arg Gly Arg Arg Asn Thr Arg Lys Gln Leu Gln Pro Ala Glu Lys Glu Ile Ala Leu His Leu Gln Ser Glu Ile Ser Ser Asp Gly Gln Lys Asp Gly Leu Asn Leu Ser Thr Ser Gln Gln Glu Ser Ile Ser Met Ile Pro Lys Gly Pro Pro Glu Asn Ser Val Ile Ser Cys Asp Ser Gln Ala Leu Asn Met Leu Ala Asp Leu Ala Leu Ser Ser Ala Ala Ala Ser Ile Pro Ser Cys Lys Pro Arg Asn Leu Pro Cys Val Ser Asp Leu Pro Arg Asn Asn Val Leu Leu Thr Lys Glu Asn Pro Leu Leu Gly Ala Ser Asp His Glu Tyr His Lys Gly

SEQ ID NO:11

Ser Ser Asp Glu Glu Glu Glu Glu Glu Asn Val Thr Cys Glu Glu Lys Ala Lys Asn Ala Asn Lys Pro

What is claimed is:

1. An isolated PBR-associated protein (PAP) DNA fragment or any portion thereof.
2. An isolated and purified DNA fragment which 5 encodes a PBR-associated protein.
3. An isolated and purified DNA fragment which encodes a peptide of PBR-associated protein, said DNA fragment comprising a sequence specified in any of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and 10 SEQ ID NO:5, or polynucleotide fragment of said sequence comprising at least 30 nucleotides.
4. An isolated and purified DNA fragment which encodes a peptide of PBR-associated protein, said DNA fragment comprising a sequence specified in Genbank 15 Accession no. AF022770, or GenBank Accession no. AF020338, or a polynucleotide fragment of said sequence comprising at least 30 nucleotides.
5. An isolated and purified PAP7 DNA fragment according to claim 2 which encodes 463 amino acids of 20 PAP7 or a natural variant or synthetic variant thereof encoding PAP7, or a peptide fragment thereof comprising at least 10 amino acids.
6. An isolated and purified PAP8 DNA fragment according to claim 2 which encodes 190 amino acids of 25 PAP8 or a natural variant or synthetic variant thereof encoding PAP8, or a peptide fragment thereof comprising at least 10 amino acids.
7. An isolated and purified PAP15 DNA fragment according to claim 2 which encodes 164 amino acids of 30 PAP15 or a natural variant or synthetic variant thereof encoding PAP15, or a peptide fragment thereof comprising at least 10 amino acids.
8. An isolated and purified PAP20 DNA fragment according to claim 2 which encodes 196 amino acids of 35 PAP20 or a natural variant or synthetic variant

thereof encoding PAP20, or a peptide fragment thereof comprising at least 10 amino acids.

9. A recombinant DNA construct comprising:

(i) a vector, and

5 (ii) the PAP DNA fragment of claim 1.

10. A recombinant DNA construct comprising:

(i) a vector, and

(ii) the PAP DNA fragment of claim 3.

11. A recombinant DNA construct according to  
10 claim 10, wherein said vector is an expression vector.

12. The recombinant DNA construct according to  
claim 10, wherein said vector is a prokaryotic vector.

13. The recombinant DNA construct according to  
claim 10, wherein said vector is a eukaryotic vector.

15 14. A host cell transformed with a recombinant  
DNA construct according to claim 10.

15 15. A host cell according to claim 14, wherein  
said cell is prokaryotic.

20 16. A host cell according to claim 14, wherein  
said cell is eukaryotic.

25 17. A method for producing PAP peptide which  
comprises culturing the cells according to either  
claim 15 or 16, under conditions such that said DNA  
fragment is expressed and said PAP peptide is thereby  
produced.

18. An isolated recombinant PAP produced by the  
method of claim 17.

30 19. A PAP7 polypeptide comprising the amino acid  
sequence specified in SEQ ID NO:7 or a portion thereof  
of at least 5 amino acids.

20. A PAP8 polypeptide comprising the amino acid  
sequence specified in SEQ ID NO:8 or a portion thereof  
of at least 5 amino acids.

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21. A PAP15 polypeptide comprising the amino acid sequence specified in SEQ ID NO:9 or a portion thereof of at least 5 amino acids.

22. A PAP20 polypeptide comprising the amino acid sequence specified in SEQ ID NO:10 or a portion thereof of at least 5 amino acids.

23. A method for detecting a PAP in a sample chosen from the group consisting of: PAP7, PAP8, PAP15, PAP20, said method comprising

10 (i) contacting said sample with antibodies which recognize said PAP; and

(ii) detecting the presence or absence of a complex formed between PAP and antibodies specific therefor.

15 24. A method for detecting a PBR-associated protein, said method comprising the two hybrid assay.

25. An antibody to a peptide having the amino acid sequence specified in SEQ ID NO:6, 7, 8 and 9, or any portion thereof.

20 26. A PAP7 antibody to a peptide comprising the amino acid sequence specified in SEQ ID NO:11.

27. A method for detecting agents or drugs which reduce or eliminate PAP activity, said method comprising:

25 (i) delivering a recombinant DNA construct according to claim 10 into a cell such that PAP is produced in said cell;

(ii) adding at least one drug or agent to said cell alone or in combination; and,

30 (iii) detecting PAP activity in said cell in the presence of said agent or drug and comparing it to a control which did not receive said drug or agent wherein a decrease in PAP activity as compared to control indicates an drug or agent which reduces or

35 eliminates PAP activity.

28. A method for detecting agents or drugs which promote PAP activity, said method comprising:

(i) delivering a recombinant DNA construct according to claim 10 into a cell such that PAP is produced in said cell;

(ii) adding at least one drug or agent to said cell alone or in combination; and,

(iii) detecting whether or not said drug or agent stimulates PAP activity by measuring PAP activity in said cell and comparing it to a control which did not receive said drug or agent wherein an increase in the activity of said PAP in said cell as compared to control indicates a stimulatory drug or agent.

29. An agent or drug capable of inhibiting PAP activity.

30. An agent or drug capable of promoting PAP activity.

31. A therapeutic compound comprising said agent or drug according to claim 29 for use in a disease wherein a decrease or elimination of PAP activity is beneficial.

32. A therapeutic compound comprising said agent or drug according to claim 30 for use in a disease wherein an increase of PAP activity is beneficial.

33. A method for detecting at least one PAP selected from the group consisting of PAP7, PAP8, PAP15, and PAP20 in a sample using the polymerase chain reaction.

34. A diagnostic kit for detecting RNA/cDNA of at least one PAP chosen from the group consisting of PAP7, PAP8, PAP15 and PAP20, in a sample comprising primers or oligonucleotides specific for said PAP RNA or cDNA suitable for hybridization to PAP RNA or cDNA and/or amplification of PAP sequences and suitable ancillary reagents.

35. A method for increasing a PAP selected from the group consisting of PAP3, PAP7, PAP8, PAP15 and PAP20 in a cell by introducing into said cell a PAP nucleic acid encoding said PAP such that said nucleic acid is expressed and PAP is produced in said cell.

5           36. A therapeutic method for the treatment or amelioration of diseases resulting from an increase in cell proliferation, said method comprising providing to an individual in need of such treatment an 10 effective amount of an agent or drug which reduces or eliminates PAP expression or function in a pharmaceutically acceptable diluent.

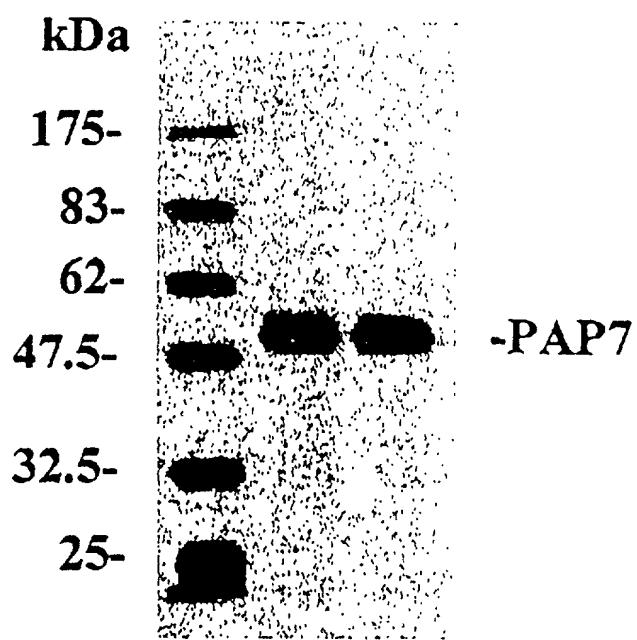
15           37. The method of claim 36 wherein said disease is cancer.

15           38. A therapeutic method for the treatment or amelioration of conditions resulting from abnormal cholesterol level, said method comprising providing to an individual in need of such treatment an effective amount of an agent or drug which reduces or eliminates 20 PAP expression or function in a pharmaceutically acceptable diluent.

25           39. The method of claim 38 wherein said condition is selected from the group consisting of: cancer, neurodegenerative disorders, developmental disorders, stress, and stroke.

30           40. A method for modulating PBR activity, function or targeting in a cell, said method comprising increasing or decreasing level of PAP in said cell.

A



B

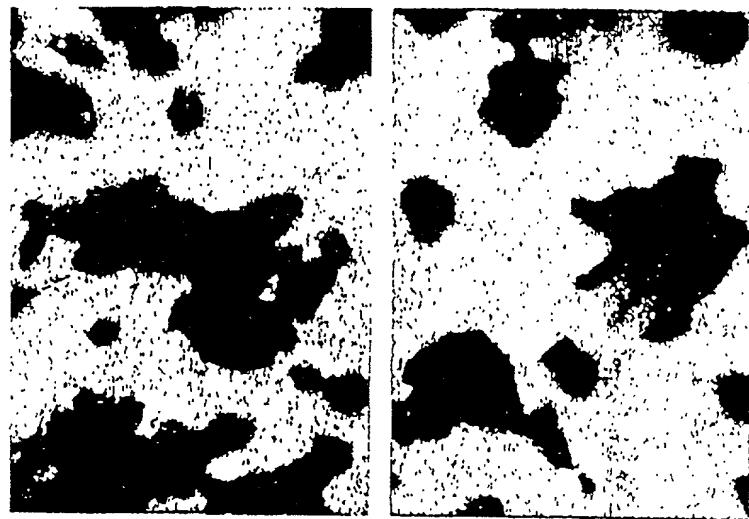


FIGURE 1/8

A



	1	2	3	4	5
A	brain	eye	liver	lung	kidney
B	heart	skeletal muscle	small intestine		
C	pancreas	thyroid	thymus	submax. gland	spleen
D	testis	ovary	prostate	epididymus	uterus
E	embryo 7 days	embryo 11 days	embryo 15 days	embryo 17 days	

B

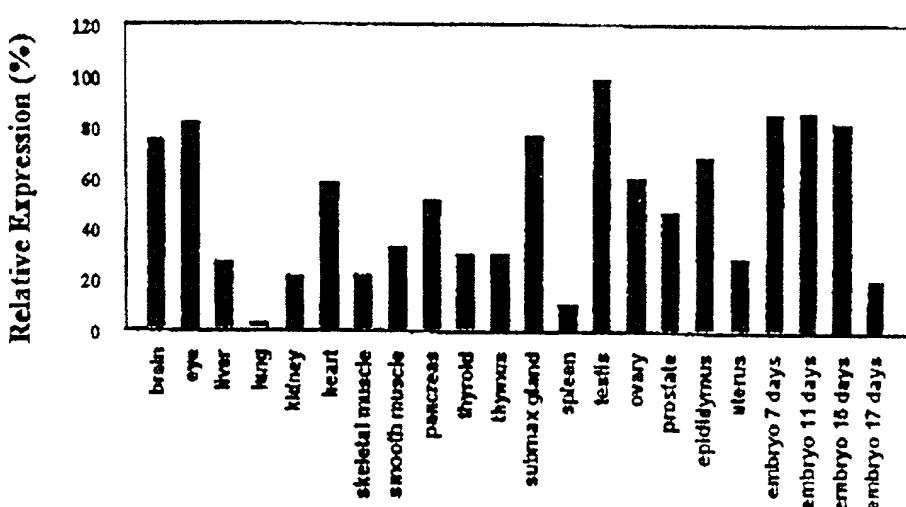


FIGURE 2/8

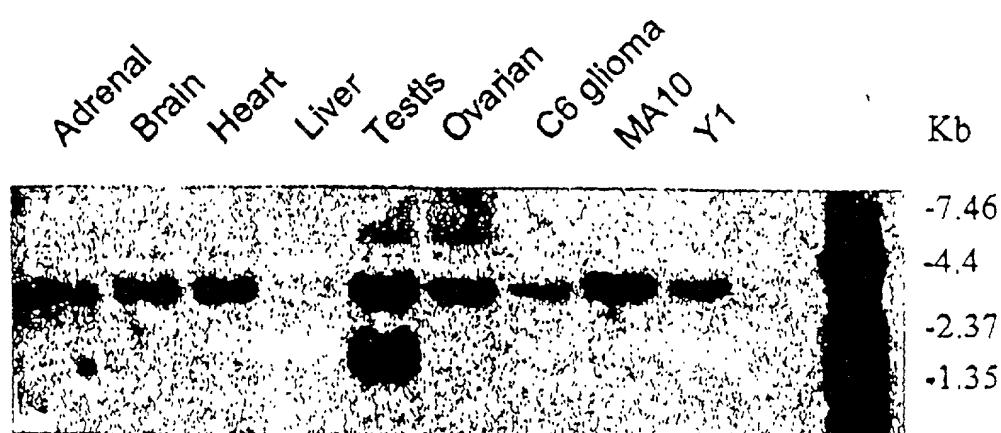
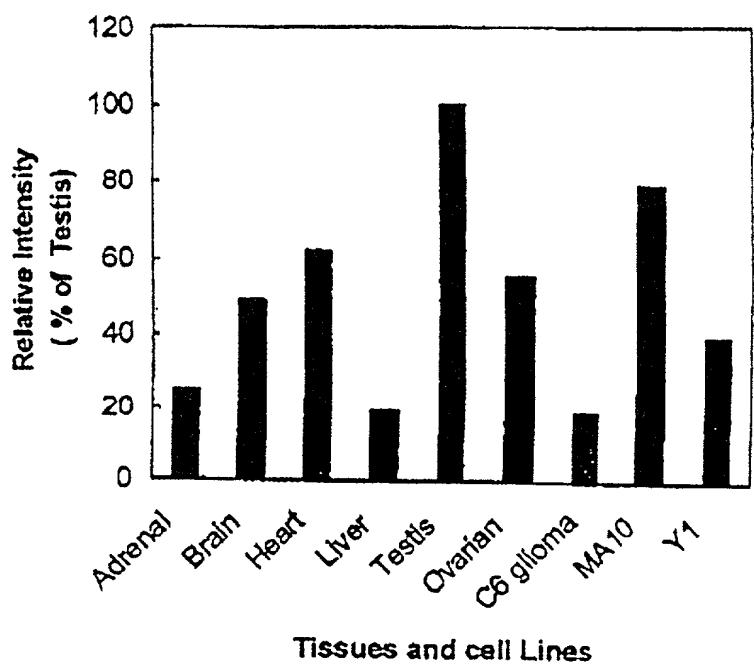
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FIGURE 3/8

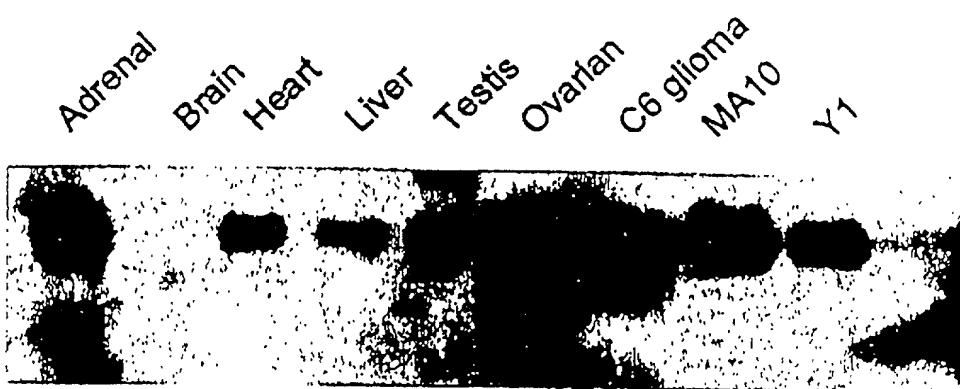
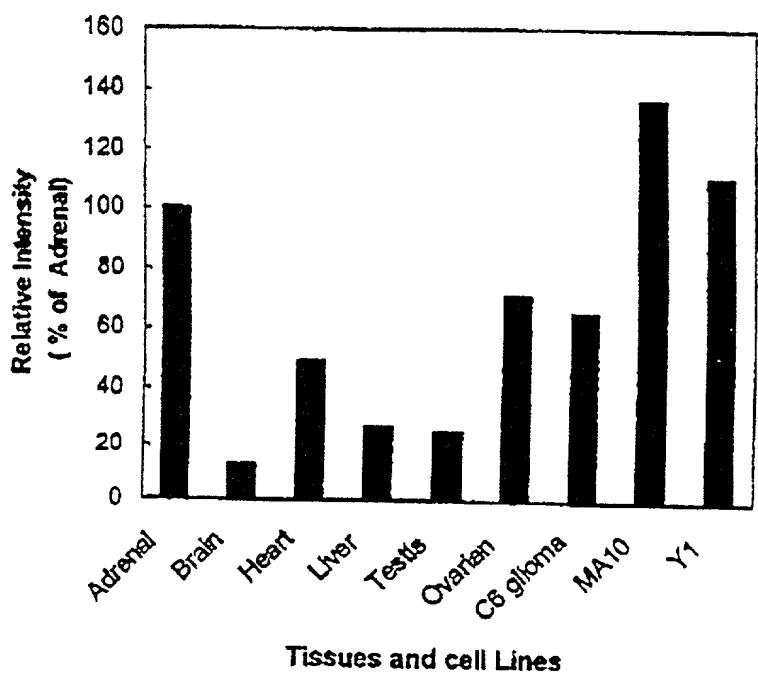
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FIGURE 4/8

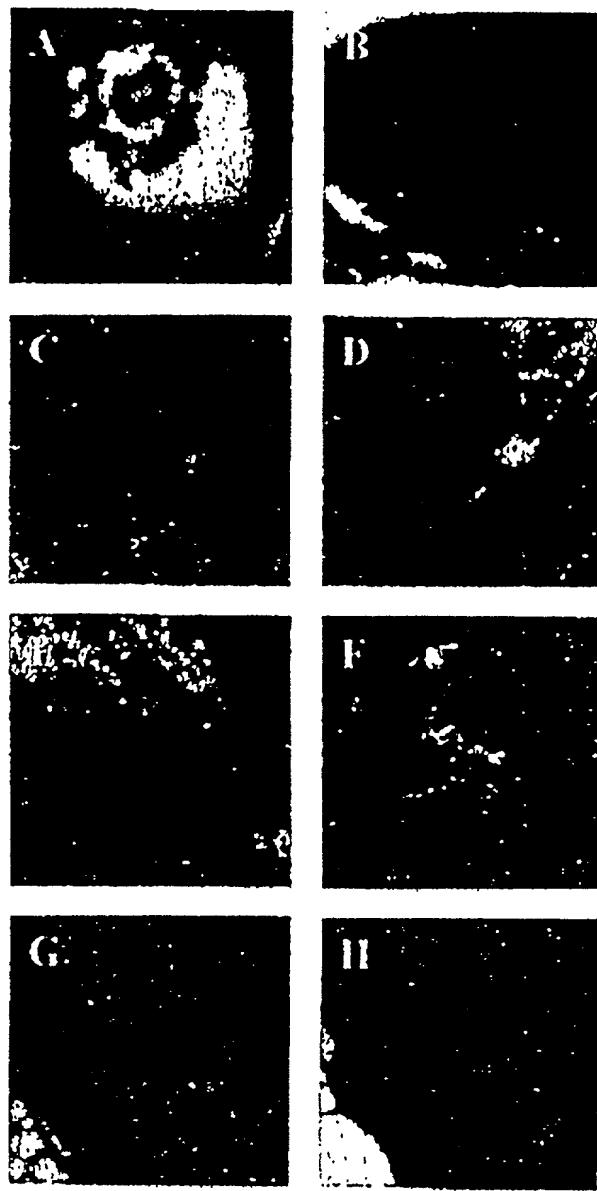
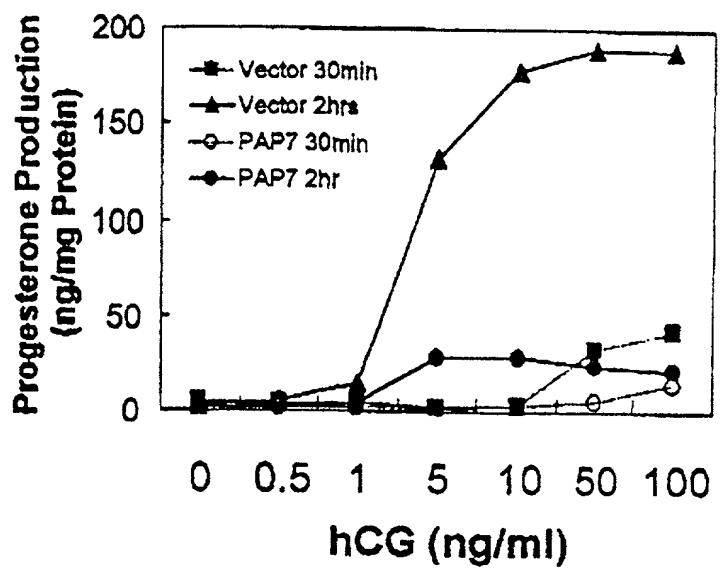


FIGURE 5/8

A



B

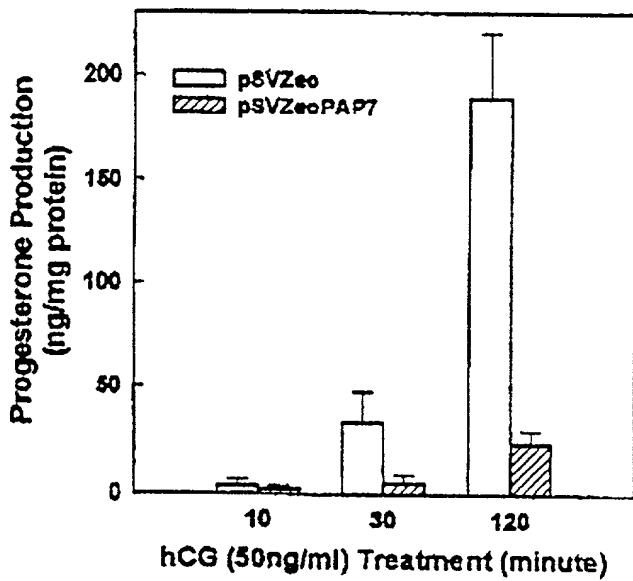


FIGURE 6/8

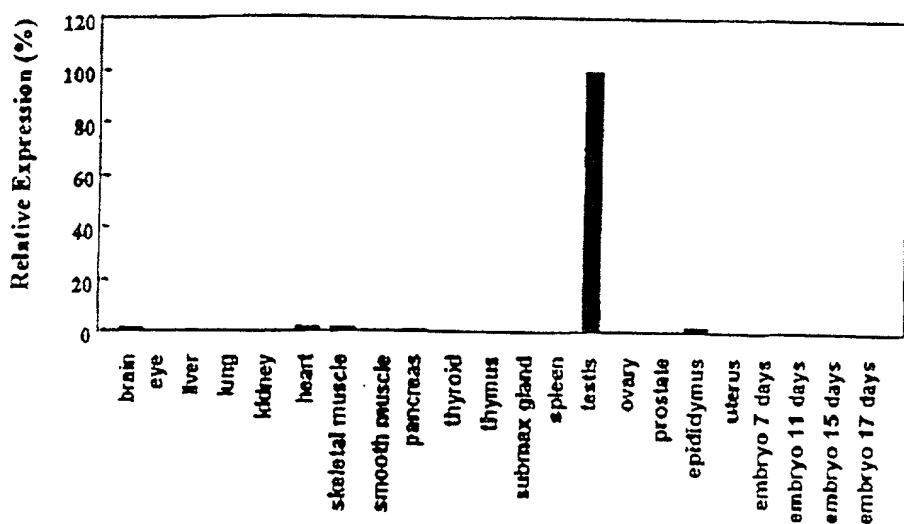
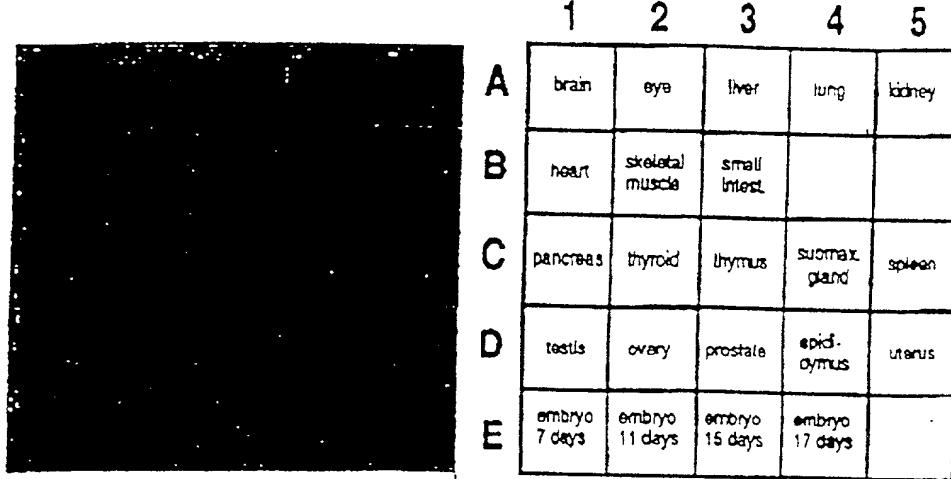
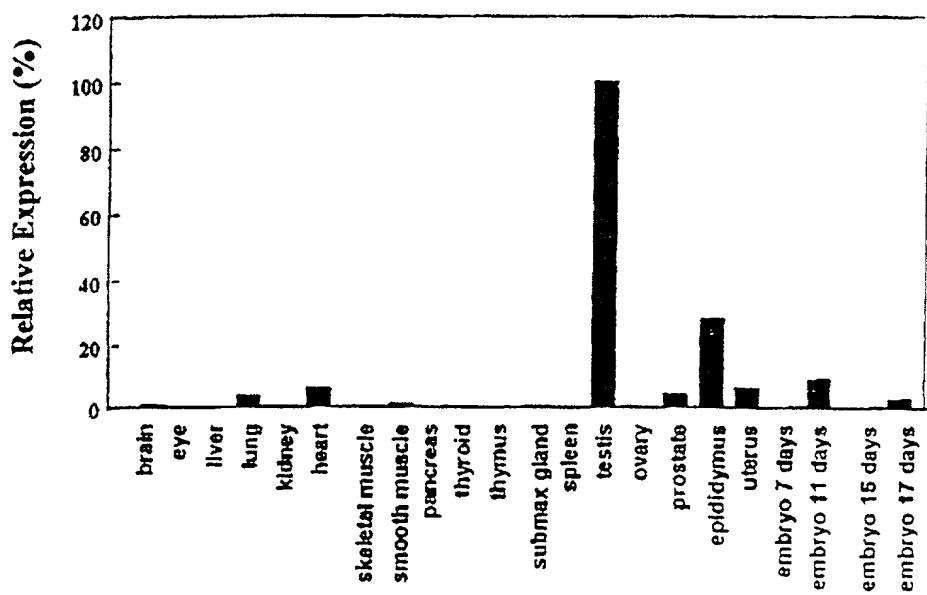
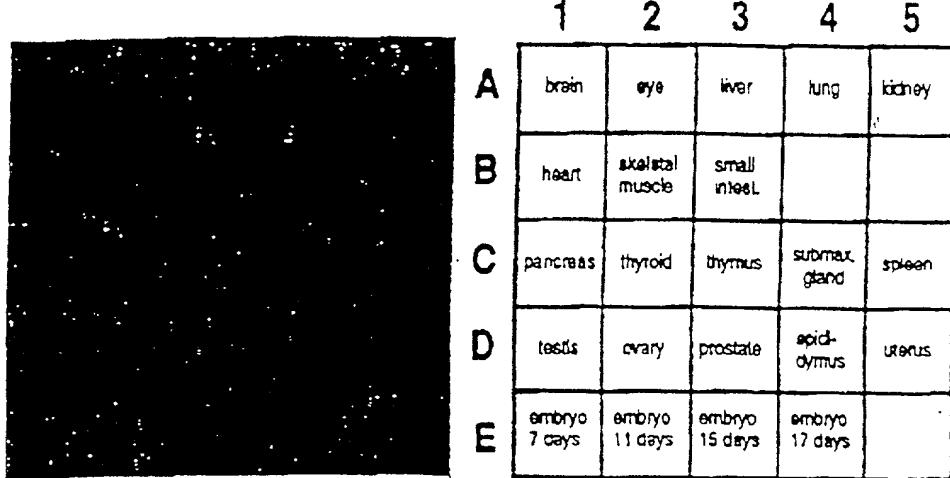


FIGURE 7/8

8 / 8



FIGURES / 8

DAD 20

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the INVENTION ENTITLED PERIPHERAL-TYPE BENZODIAZEPINE RECEPTOR ASSOCIATED PROTEINS, CLONING, EXPRESSION AND METHODS OF USE

the specification of which (CHECK applicable BOX(ES))  
A.  is attached hereto.

BOX(ES) → B.  was filed on \_\_\_\_\_ as U.S. Application No. \_\_\_\_\_ /  
→ C.  was filed as PCT International Application No. PCT/ US99/18507 on August 11, 1999

and (if applicable to U.S. or PCT application) was amended on \_\_\_\_\_

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 C.F.R. 1.56. Except as noted below, I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International Application which designated at least one other country than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate, or PCT International Application, filed by me or my assignee disclosing the subject matter claimed in this application and having a filing date (1) before that of the application on which priority is claimed, or (2) if no priority claimed, before the filing date of this application:

PRIOR FOREIGN APPLICATION(S)		Date first Laid-open or Published	Date Patented or Granted	Priority NOT Claimed
Number	Country	Day/MONTH/Year Filed		
PCT/US99/18507	International	11 August 1999	24 February 2000	

If more prior foreign applications, X box at bottom and continue on attached page.

Except as noted below, I hereby claim domestic priority benefit under 35 U.S.C. 119(e) or 120 and/or 365(c) of the indicated United States applications listed below and PCT international applications listed above or below and, if this is a continuation-in-part (CIP) application, insofar as the subject matter disclosed and claimed in this application is in addition to that disclosed in such prior applications, I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 C.F.R. 1.56 which became available between the filing date of each such prior application and the national or PCT international filing date of this application:

PRIOR U.S. PROVISIONAL, NONPROVISIONAL AND/OR PCT APPLICATION(S)		Status	Priority NOT Claimed
Application No. (series code/serial no.)	Day/MONTH/Year Filed		
60/096,048	11 August 1998	pending, abandoned, patented	pending

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

And I hereby appoint Pillsbury Winthrop LLP, Intellectual Property Group, 1100 New York Avenue, N.W., Ninth Floor, East Tower, Washington, D.C. 20005-3918, telephone number (202) 861-3000 (to whom all communications are to be directed), and the below-named persons (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent, and I hereby authorize them to delete names/numbers below of persons no longer with their firm and to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct the above Firm and/or a below attorney in writing to the contrary.

Paul N. Kokulis	16773	Kendrew H. Colton	30368	Roger R. Wise	31204	Anthony L. Miele	34393
G. Lloyd Knight	17698	G. Paul Edgell	24238	Michael R. Dzwonczyk	36787	Robert J. Walters	40862
Kevin E. Joyce	20508	Lynn E. Eccleston	35861	W. Patrick Bengtsson	32456	Brian J. Beatus	38825
George M. Sirilla	18221	Timothy J. Klima	34852	Jack S. Barufka	37087		
Donald J. Bird	25323	David A. Jakopin	32995	Adam R. Hess	41835		
Dale S. Lazar	28872	Mark G. Paulson	30793	William P. Atkins	38821		
Paul E. White, Jr.	32011	Stephen C. Glazier	31361	Paul L. Sharer	36004		
Glenn J. Perry	28458	Richard H. Zaitlen	27248	Robin L. Teskin	35030		

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"X" box  FOR ADDITIONAL INVENTORS, and proceed on the attached page to list each additional inventor.

See additional foreign priorities on attached page (incorporated herein by reference).

Atty. Dkt. No. P0277848

(M#)

## SEQUENCE LISTING

<110> PAPADOPoulos, VASSILIOS  
HAU, LI

<120> PERIPHERAL-TYPE BENZODIAZEPINE RECEPTOR ASSOCIATED  
PROTEINS, CLONING, EXPRESSION AND METHODS OF USE

<130> 082137-0277848

<140> 09/762,594  
<141> 2001-02-09

<150> PCT/US99/18507  
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aattgctaat aaggcctctg tagccatggc tacttctgac gtgaaaccaa aatcaataag 120  
tcgtgccaag aaatggtcag agggaaataga aaatctgtac agatttcaac aagcaggata 180  
tcgggatgaa attgaatata aacaagtgaa acaagttgcc atggtcgacc gatggccaga 240  
gacagggtac gtgaagaaac ttcaagcgag ggacaatact ttcttctact acaacaaaaga 300  
gagggagatgc gaggacaagg aggtccacaa agtgaagggt tacgtctact gacctttcc 360  
tttcttcggc ttggcaatgc tccttaaga attgggtgtt tacattttc catcgtgtaa 420  
atgtcatttt acaaaaacaat tcacaattct gtcttaatt catggtgtct tacacaacat 480  
aaacaccac cttgaaaccc aaaaa 505

<210> 2  
<211> 1459  
<212> DNA  
<213> Mus musculus

<400> 2  
gaattcgcgg ccgcgtcgac ctaaagtgtga gttgttcact gtagtgaccc gtgtgaaggt 60  
agttttatatt ttaaatcaac ttcatgtgt ccaaactagta aaagatggca aagctttca 120  
tccaacttat gaagaaaaac tgaagttcgt ggcactgcat aagcaagttc ttttgggccc 180  
atataaccca gacacgtccc ctgaggttgg attctttgtat gtgttgggga atgataggag 240  
gagagaatgg gcagctctgg gaaacatgtc caaggaggat gccatggtag agtttgtgaa 300  
gcttctaaat aagtgttgc ctctcctctc ggcataatgtt gctcccaca gaatagagaa 360  
ggaagaagaa gagaaaaagaa gaaaggcgga ggaggagcga aggcagcgtg aagaggaaga 420  
acgagagcgg ctgcaaaagg aagaagagaa gcgaaagcga gaggaggaaag accggctgag 480  
acggggaggag gaagagaggc ggcggataga ggaagagagg cttcggctgg aacagcaaaa 540  
gcagcagata atggcagtt taaactcgca gactgccgtg caattccagc agtatgcagc 600  
ccagcagat ccagggact acgaacaaca gcagattctc atccgcccagc tgcaggagca 660  
gcactatcag cagtataaac accaggcaga gcaaacccaa cctgcacaac aacaggcagc 720

attacagaaa cagcaagaag tagtgatggc tggggcatca ttgcctgcat catcaaagg 780  
 gaacacagt ggagaaggatc atacactgtc agttaatggc caggccaaa cccacactga 840  
 aaattccgaa aaagtccttg agccagaagc tgcagaagaa gccttggaaa atgaccaaa 900  
 agactctttt ccagtgattt cagctccatc catgtggaca agaccacaaa tcaaagactt 960  
 taaaagagaag attccggcagg atgcagattc tgcatttgc gtaatggata gatacgatcgt 1020  
 caccgtccga gtcccgactc atgagggagg atcataccta ttttggaaat ttgccacaga 1080  
 cagttatgac attgggtttt gggtttattt tgaatggaca gactctccaa atgctgctgt 1140  
 cagtgtgcat gtcagtgagt ccagtgcga ggaggaggag gaggaagaaa atgtcacttg 1200  
 tgaagaaaaa gcaaaaaaaga acgccaacaa gcctctgctg gatgagattt tacatgtgta 1260  
 ccggcgggac tgcacgagg aagtataatgc aggccac cgtatccag ggagggggagt 1320  
 ctatctccctc aagtttgata attcctactc tctgtggagg tccaaatccg tctactacag 1380  
 agtcttattat actagataga gctgctttc caaggtccgg agtccagggt tgagcacaac 1440  
 atgacgttta atttccttt 1459

<210> 3  
 <211> 568  
 <212> DNA  
 <213> Mus musculus

<400> 3  
 gaattcgcgg ccgcgtcgac gctggacaca agcgtggagc gaagagccct tggagagatt 60  
 cagaatgtag gtgaaggctc ttcaacatca cagggcacct ggcagtcttc agagtccctca 120  
 cagtccaaacc tgggggagca gacgcagagc ggaccccagg gaggaaggtg tcagcgtcgg 180  
 gagaggcata accgaatggc acgagatagg aggcgcagaa tccgcatttgc ctgtgatgag 240  
 ctgaatctt tagtccatt ctgcaatgcg gagacagata aagcaacaac ccttcagtgg 300  
 accacagcat tcctgaagta cattcagggaa agacatgggg actctcttaa aaaggaattt 360  
 gagagcgtgt tttgcggtaa aactggcaga aggttaaagc tgactagacc cgaatccctg 420  
 gtgacctgcc ctgcacaggc cagcctgcag agcagccctg ccatggagat caagtgactg 480  
 gactgaccca ggacctggga gagaaccgc gttcctgcgg catcatgcac atgcctgcca 540  
 tccccggaaat tcagctctga atcctctc 568

<210> 4  
 <211> 490  
 <212> DNA  
 <213> Mus musculus

<400> 4  
 gaattcgcgg ccgcgtcgac ctcggggta acccttggga tcattatgtat gtcacctctt 60  
 caatctcttgc agaccagtga tttatgaact tgacatttgg tgcctgggtgt gggctttgg 120  
 aagcagaggc cacctttgtc tgcgtggat actgagccgc tggatggcag gaatccaaag 180  
 agaagccagc ctctcgtagt cgccttggc cagttggaga gagggaggac cggcaaaagg 240  
 gaatctgtct ttctccctagg cccgagcatg tccctgtgg gacatgtct gtgacagctg 300  
 agcctgcccgc gcctgcctt ctgaagttgg gtgtctccctg cccacaacca agccagcaat 360  
 cggtctgttt tccgacaacc tcagagccag acctcacaag cctatttgg tggttcccaa 420  
 aatttctctc agatctccat gtctatccct ccactccctc caaaagagaa agaaaagaat 480  
 tgagaaagaa 490

<210> 5  
 <211> 588  
 <212> DNA  
 <213> Mus musculus

<400> 5  
 gaattcgcgg ccgcgtcgac ctcagaagag gaaaagaggt gcagaagtgc tggcggcaca 60  
 aattgtacag aaaaccagac tagagggaaa aaaacaagaa gcgtctgtat ctaaagatgc 120

tccagtgcct acaaatacta aaagggcaaa gaaacaagag aagtctccag gtagaattgc 180  
 ctcacagtct aagccaccca tgaaaaagtc tccacaaaaa cggaaggtaa attagcaag 240  
 aggccgtcgg aataccagaa agcagctcca acctgcccga aaagaaaattg ctttacatct 300  
 tcaatcagaa atttcatcag atggccaaaa agatggactt aacctaagta catctcaaca 360  
 agaaaagtatt tcaatgattc ctaaagggtcc tcctgaaaac tcagttatca gctgtgactc 420  
 ccaggcccta aatatgttag ctgatctggc attaagttct gctgctgctt ctataccatc 480  
 ttgttaagccc aggaaccttc cctgcgtctc tgatttgcca cgaaacaatg tcttactcac 540  
 taaagaaaaat ccattgcttg gtgcctctga ccatgaatat cataaggg 588

<210> 6  
 <211> 88  
 <212> PRT  
 <213> Mus musculus

<400> 6  
 Met Ala Thr Ser Asp Val Lys Pro Lys Ser Ile Ser Arg Ala Lys Lys  
 1 5 10 15

Trp Ser Glu Glu Ile Glu Asn Leu Tyr Arg Phe Gln Gln Ala Gly Tyr  
 20 25 30

Arg Asp Glu Ile Glu Tyr Lys Gln Val Lys Gln Val Ala Met Val Asp  
 35 40 45

Arg Trp Pro Glu Thr Gly Tyr Val Lys Lys Leu Gln Arg Arg Asp Asn  
 50 55 60

Thr Phe Phe Tyr Tyr Asn Lys Glu Arg Glu Cys Glu Asp Lys Glu Val  
 65 70 75 80

His Lys Val Lys Val Tyr Val Tyr  
 85

<210> 7  
 <211> 463  
 <212> PRT  
 <213> Mus musculus

<400> 7  
 Arg Pro Arg Arg Pro Lys Val Glu Leu Phe Thr Val Val Thr Arg Val  
 1 5 10 15

Lys Val Val Leu Phe Leu Asn Gln Leu Ser Leu Cys Lys Leu Val Lys  
 20 25 30

Asp Gly Lys Ala Phe His Pro Thr Tyr Glu Glu Lys Leu Lys Phe Val  
 35 40 45

Ala Leu His Lys Gln Val Leu Leu Gly Pro Tyr Asn Pro Asp Thr Ser  
 50 55 60

Pro Glu Val Gly Phe Phe Asp Val Leu Gly Asn Asp Arg Arg Arg Glu  
 65 70 75 80

Trp Ala Ala Leu Gly Asn Met Ser Lys Glu Asp Ala Met Val Glu Phe  
 85 90 95

Val Lys Leu Leu Asn Lys Cys Cys Pro Leu Leu Ser Ala Tyr Val Ala  
 100 105 110

Ser His Arg Ile Glu Lys Glu Glu Glu Lys Arg Arg Lys Ala Glu  
 115 120 125

Glu Glu Arg Arg Gln Arg Glu Glu Glu Arg Glu Arg Leu Gln Lys  
 130 135 140

Glu Glu Glu Lys Arg Lys Arg Glu Glu Asp Arg Leu Arg Arg Glu  
 145 150 155 160

Glu Glu Glu Arg Arg Arg Ile Glu Glu Glu Arg Leu Arg Leu Glu Gln  
 165 170 175

Gln Lys Gln Gln Ile Met Ala Ala Leu Asn Ser Gln Thr Ala Val Gln  
 180 185 190

Phe Gln Gln Tyr Ala Ala Gln Gln Tyr Pro Gly Asn Tyr Glu Gln Gln  
 195 200 205

Gln Ile Leu Ile Arg Gln Leu Gln Glu Gln His Tyr Gln Gln Tyr Lys  
 210 215 220

His Gln Ala Glu Gln Thr Gln Pro Ala Gln Gln Ala Ala Leu Gln  
 225 230 235 240

Lys Gln Gln Glu Val Val Met Ala Gly Ala Ser Leu Pro Ala Ser Ser  
 245 250 255

Lys Val Asn Thr Ala Gly Ala Ser Asp Thr Leu Ser Val Asn Gly Gln  
 260 265 270

Ala Lys Thr His Thr Glu Asn Ser Glu Lys Val Leu Glu Pro Glu Ala  
 275 280 285

Ala Glu Glu Ala Leu Glu Asn Gly Pro Lys Asp Ser Leu Pro Val Ile  
 290 295 300

Ala Ala Pro Ser Met Trp Thr Arg Pro Gln Ile Lys Asp Phe Lys Glu  
 305 310 315 320

Lys Ile Arg Gln Asp Ala Asp Ser Val Ile Thr Val Arg Arg Gly Glu  
 325 330 335

Val Val Thr Val Arg Val Pro Thr His Glu Glu Gly Ser Tyr Leu Phe  
 340 345 350

Trp Glu Phe Ala Thr Asp Ser Tyr Asp Ile Gly Phe Gly Val Tyr Phe  
 355 360 365

Glu Trp Thr Asp Ser Pro Asn Ala Ala Val Ser Val His Val Ser Glu  
 370 375 380

Ser Ser Asp Glu Glu Glu Glu Glu Asn Val Thr Cys Glu Glu  
 385 390 395 400

Lys	Ala	Lys	Lys	Asn	Ala	Asn	Lys	Pro	Leu	Leu	Asp	Glu	Ile	Val	Pro
								405			410				415
Val	Tyr	Arg	Arg	Asp	Cys	His	Glu	Glu	Val	Tyr	Ala	Gly	Ser	His	Gln
									420		425				430
Tyr	Pro	Gly	Arg	Gly	Val	Tyr	Leu	Leu	Lys	Phe	Asp	Asn	Ser	Tyr	Ser
								435		440					445
Leu	Trp	Arg	Ser	Lys	Ser	Val	Tyr	Tyr	Arg	Val	Tyr	Tyr	Thr	Arg	
									450		455				460

<210> 8  
<211> 158  
<212> PRT  
<213> *Mus musculus*

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<400> 8
Glu Phe Ala Ala Ala Ser Thr Leu Asp Thr Ser Val Glu Arg Arg Ala
1 5 10 15

Leu Gly Glu Ile Gln Asn Val Gly Glu Gly Ser Ser Thr Ser Gln Gly
20 25 30

Thr Trp Gln Ser Ser Glu Ser Ser Gln Ser Asn Leu Gly Glu Gln Thr
35 40 45

Gln Ser Gly Pro Gln Gly Gly Arg Cys Gln Arg Arg Glu Arg His Asn
50 55 60

Arg Met Glu Arg Asp Arg Arg Arg Arg Ile Arg Ile Cys Cys Asp Glu
65 70 75 80

Leu Asn Leu Leu Val Pro Phe Cys Asn Ala Glu Thr Asp Lys Ala Thr
85 90 95

Thr Leu Gln Trp Thr Thr Ala Phe Leu Lys Tyr Ile Gln Glu Arg His
100 105 110

Gly Asp Ser Leu Lys Lys Glu Phe Glu Ser Val Phe Cys Gly Lys Thr
115 120 125

Gly Arg Arg Leu Lys Leu Thr Arg Pro Glu Ser Leu Val Thr Cys Pro
130 135 140

Ala Gln Gly Ser Leu Gln Ser Ser Pro Ala Met Glu Ile Lys
145 150 155

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<210> 9  
<211> 112  
<212> PRT  
<213> *Mus musculus*

<400> 9  
Ala Ala Gly Trp Gln Glu Ser Lys Glu Lys Pro Ala Ser Arg Ser Arg  
1 5 10 15

Pro Gly Thr Val Glu Glu Arg Glu Asp Arg Gln Arg Gly Ile Cys Leu  
     20                 25                 30

Ser Pro Arg Pro Glu His Val Pro Cys Gly Thr Cys Ser Val Thr Ala  
     35                 40                 45

Glu Pro Ala Gln Pro Ala Phe Leu Lys Leu Gly Val Ser Cys Pro Gln  
     50                 55                 60

Pro Ser Gln Gln Ser Val Cys Phe Pro Thr Thr Ser Glu Pro Asp Leu  
     65                 70                 75                 80

Thr Ser Leu Phe Trp Trp Phe Pro Lys Phe Leu Ser Asp Leu His Val  
     85                 90                 95

Tyr Pro Ser Thr Pro Ser Lys Arg Glu Arg Lys Glu Leu Arg Lys Lys  
     100                105                110

<210> 10  
 <211> 196  
 <212> PRT  
 <213> Mus musculus

<400> 10  
 Asn Ser Arg Pro Arg Arg Pro Gln Lys Arg Lys Arg Gly Ala Glu Val  
     1                 5                 10                 15

Leu Ala Ala Gln Ile Val Gln Lys Thr Arg Leu Glu Arg Lys Lys Gln  
     20                25                 30

Glu Ala Ser Val Ser Lys Asp Ala Pro Val Pro Thr Asn Thr Lys Arg  
     35                40                 45

Ala Lys Lys Gln Glu Lys Ser Pro Gly Arg Ile Ala Ser Gln Ser Lys  
     50                55                 60

Pro Pro Met Lys Lys Ser Pro Gln Lys Arg Lys Val Asn Val Ala Arg  
     65                70                 75                 80

Gly Arg Arg Asn Thr Arg Lys Gln Leu Gln Pro Ala Glu Lys Glu Ile  
     85                90                 95

Ala Leu His Leu Gln Ser Glu Ile Ser Ser Asp Gly Gln Lys Asp Gly  
     100              105                 110

Leu Asn Leu Ser Thr Ser Gln Gln Glu Ser Ile Ser Met Ile Pro Lys  
     115              120                 125

Gly Pro Pro Glu Asn Ser Val Ile Ser Cys Asp Ser Gln Ala Leu Asn  
     130              135                 140

Met Leu Ala Asp Leu Ala Leu Ser Ser Ala Ala Ala Ser Ile Pro Ser  
     145              150                 155                 160

Cys Lys Pro Arg Asn Leu Pro Cys Val Ser Asp Leu Pro Arg Asn Asn  
165 170 175  
Val Leu Leu Thr Lys Glu Asn Pro Leu Leu Gly Ala Ser Asp His Glu  
180 185 190  
Tyr His Lys Gly  
195

<210> 11  
<211> 25  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: synthetic  
peptide

<400> 11  
Ser Ser Asp Glu Glu Glu Glu Glu Asn Val Thr Cys Glu Glu  
1 5 10 15  
Lys Ala Lys Lys Asn Ala Asn Lys Pro  
20 25